Evaluation of GIP and Intestinal GLP-1(7-36)amide Function in Hyperphagic States

A Thesis presented for the degree of Doctor of Philosophy
by
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For David & my parents.
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SUMMARY

The importance of two entero-endocrine hormones, namely GIP and GLP-1(7-36)amide, in a range of physiological and pathological states associated with hyperphagia, was investigated in rodents. The states investigated consisted of pregnancy, lactation, cold acclimation, modified suckling neonatal nutrition, insulinoma, streptozotocin diabetes and the genetic diabetic syndromes of ob/ob mice and BB rats. In addition, the ontogeny of the two hormones was monitored and the importance of the maternal diet to the physiological hyperinsulinaemia of the foetus investigated.

Plasma and/or intestinal IR-GIP were shown to increase in all hyperphagic conditions with the exception of insulinoma. In all groups with increased basal plasma GIP concentrations, levels were reduced towards control values by an 18 hour fast. In the pathological states of insulinoma and streptozotocin, during suckling neonatal overnutrition and after two weeks of cold acclimation, the GIP response to acute glucose or fat stimulation was exaggerated. However, following weaning the overnourished animals had normal plasma and intestinal GIP concentrations, and after five weeks cold acclimation the plasma GIP response to acute glucose stimulation was normalised. A strong correlation between hyperphagia and increased K cell stimulation was therefore concluded. GLP-1(7-36)amide concentrations were monitored in the intestine. A relationship between intestinal GLP-1(7-36)amide concentration and hyperphagia was suggested from several of the studies which demonstrated an increased intestinal GLP-1(7-36)amide content and/or concentration. However, one of the most potent stimulators of GLP-1(7-36)amide concentration was undernutrition during the suckling period. Intestinal changes measured at this time persisted following weaning. The importance of insulin on normal K cell function was revealed by the pathological states of excessive or depleted insulin concentrations (insulinoma and streptozotocin diabetes respectively). The importance of a negative feedback
mechanism on K cell secretion was revealed in the streptozotocin study. This feedback was shown to operate on both postprandial carbohydrate and fat stimulation of the K cell. GLP-1(7-36)amide was shown to be a important component of the entero-insular axis in lean and ob/ob mice, on a relative molar basis GLP-1(7-36)amide being more potent than GIP in stimulating insulin secretion. However, an in vitro gut sac incubation technique cast doubt on whether this hormone is a true incretin as it was not directly stimulated by nutrient absorption. In addition to the effects of GIP and GLP-1(7-36)amide on insulin homeostasis, the ontogeny and neonatal studies suggested an important role for these hormones in early neonatal development. It is suggested that in addition to their actions on the B cells of the pancreas these hormones may well be important, directly and through interaction with insulin, in adipose tissue metabolism.
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INRODUCTION
1.1 The Control of Insulin Secretion

Glucose, under normal physiological circumstances, is the main metabolic fuel of the body and is widely regarded as the primary stimulus for insulin secretion from the B cells of the pancreas. Using the in-vitro technique of incubating collagenase-isolated mouse islets it can be shown that a sigmoidal relationship exists between extracellular glucose concentration and insulin secretion ($\frac{1}{2}V_{max}$) requiring 8mM glucose in the mouse and 6mM in the rat (Ashcroft et al., 1972). Metabolism of glucose is a necessary prerequisite for insulin secretion (Ashcroft, 1980). The B cells therefore function optimally over the normal physiological plasma glucose concentration range of these animals.

Experiments in rats have demonstrated that normal, moderate, post-prandial increases in plasma glucose, from 4.0 to 6.6 mM over 20 minutes, are a significant stimulus for plasma insulin release. Intravenous infusion of glucose to mimic similar increases in plasma glucose have shown however that this physiologically normal increase in plasma glucose produces a very weak plasma insulin response (Louis-Sylvestre, 1978). It can therefore be concluded that although the B cell is functioning within the normal, narrow range of plasma glucose concentrations, this physiological rise in plasma glucose is not, on its own, a potent stimulus for insulin secretion. Experiments in man using equal glucose loads orally and intravenously have shown similar results, a moderate insulin response to intravenous glucose, even when plasma glucose exceeds levels normally seen post-prandially (McIntyre et al., 1964).

It is recognised that insulin biosynthesis and secretion is regulated by a complex interaction of circulating nutrients, neurotransmitters, paracrine agents and gut hormones. The role of the various secretagogues alters in importance under different physiological situations (Malaisse, 1972; Malaisse, 1988). The secretory response of the B cells is therefore modulated by a number of compounds. The B cell must have
the ability to not only detect these secretagogues but also to integrate the information provided by each one. Evidence suggests that the concentration of glucose in the interstitial fluid surrounding islet cells is the principal regulator (or initiator) of insulin secretion but that neural and hormonal components of the entero-insular axis play a key role in modulating (potentiating) the secretory response to feeding (Morgan et al., 1988; Howell, 1990).

1.2 The Entero-Insular Axis

The term *entero-insular axis* was first defined by Unger & Eisentraut in 1969, as “the regulatory control exerted by the gastrointestinal tract on the endocrine pancreas.” The term encompasses neural and hormonal actions as well as direct substrate stimulation of all cells within the islets of Langerhans. Its physiological importance is demonstrated by the increased tolerance of an oral glucose load (McIntyre et al., 1964; Elrick et al., 1964; Louis-Sylvestre, 1978).

1.2.1 A Historical Perspective of the Entero-Insular Axis

In 1906, it was first suggested that oral administration of acid extracts of porcine proximal small intestine could modify the glycosuria of clinical diabetics (Moore et al., 1906). In the late 1920's and early 1930's, LaBarre and colleagues demonstrated that an endocrine factor, secreted from the gut, enhanced pancreatic insulin secretion. This factor was not the hormone secretin that they were currently investigating (LaBarre & Still, 1930). As a result of this work, LaBarre coined the term *incretin* to describe a humoral activity (or activities) of the gut that enhances endocrine secretions of the pancreas. Eight years later the idea of a duodenal hormonal control on the pancreas was rejected by Loew et al., (1940). Their experiments with fasted dogs denied the existence of a hypoglycaemic factor in the duodenum.
The development of radioimmunoassay (RIA), in the early 1960’s (Yalow and Berson, 1960), stimulated new interest in the area of gut hormones as plasma insulin itself could now be measured. Insulin RIA demonstrated for the first time that orally administered glucose evokes a greater insulin response than comparable amounts of glucose given by the intravenous route (McIntyre et al., 1964; Elrick et al., 1964). This increased insulin response was initially ascribed to enhanced insulin production, not decreased insulin turnover (Lickley et al., 1975; McIntyre et al., 1970). Comparison of insulin and C-peptide responses to oral and intravenous glucose loads however, indicate that the incretin effect is the result of both an increased insulin secretion from the B cells and decreased hepatic extraction in normal subjects (Gibby & Hales, 1983; Hampton et al., 1986; Shuster et al., 1988).

1.2.2 The Components of the Entero-Insular Axis

The enteric signal of the entero-insular axis (EIA) responsible for augmentation of insulin release was initially presumed to be one or more gastrointestinal hormones (Creutzfeldt et al., 1970; Rehfeld, 1972). The EIA is now acknowledged to consist of a neural as well as an endocrine component. This neural component has largely been overlooked because endocrine factors are easier to manipulate and quantify. Increasingly it has been realised that many gastrointestinal peptides are present in both the central nervous system and the gastrointestinal tract and evidence is accumulating that many peptides may function as neurotransmitters. This has led to an increased interest in the neural arm of the EIA. The relative importance of the two branches of the EIA have been assessed in rats by sham feeding and atropine treatment (Berthoud, 1984). From this study it was estimated that neurally mediated secretion accounted for at least 26%, and hormone mediated secretion at least 30%, of the total insulin response to a liquid test meal.
Pancreatic transplantation studies have produced conflicting results. Some studies have shown preservation of the incretin effect in pigs despite total loss of the neural component (Lindkaer-Jensen et al., 1976; Nauck et al., 1985). Other workers have shown complete abolition of the EIA (Jakob et al., 1970). The discrepancies may possibly be explained by differences in the methodology of the studies regarding the site of transplantation and venous drainage. In man, a study on diabetic patients who received a combined kidney and pancreas transplant following renal failure, showed a qualitatively preserved insulin response to oral glucose (Clarke et al., 1989).

The importance of hormonal factors under transplantation circumstances may be overestimated, possibly due to their compensation for the denervation of the pancreas, and may therefore not reflect the normal physiological situation.

Several candidate incretins have been proposed for the endocrine component of the EIA.

1.2.3 Criteria for Incretin Candidates

Several gastrointestinal hormones whose actions are endocrine in nature are able to stimulate insulin secretion in response to glucose and amino acids (Rushakoff et al., 1990). In order to be considered an incretin several criteria must be met. These criteria are listed in Table 1.1.
Table 1.1  **Criteria for Potential Incretin Candidates**

| **The hormone should be produced by gut epithelial cells and released in response to the ingestion of nutrients, especially glucose.** |
|**Exogenous administration of a pure preparation of the hormone should augment insulin release at the concentrations that occur in vivo.** |
| **Inhibition of hormone action by the use of agonists or a blocking antibody should reduce insulin secretion in response to oral nutrients.** |
| **Receptors for the hormone should be present on pancreatic B cells.** |

Several gut hormones have been studied as possible incretins, the evidence for the well established gut hormones has been reviewed by Kreymann & Bloom (1991), and Morgan (1992). Of the hormones characterised and found to be capable of promoting insulin secretion, the most interesting so far isolated are known by the acronyms GIP and GLP-1(7-36)amide. Until the discovery of GLP-1(7-36)amide during the early 1980’s, GIP was widely regarded as the major endocrine component of the EIA. It was thought to be the peptide with the most potent insulin stimulating activity secreted in response to glucose (Creutzfeldt & Ebert, 1985).

Each of these peptides will be considered separately for completeness.

1.3  **GIP**

The original name of this hormone was gastric inhibitory polypeptide as it was first characterised in terms of its gastric acid inhibitory properties. As the physiological functions of the hormone became established it was renamed, retaining the acronym, as glucose dependent insulinogetic polypeptide or glucose dependent insulin releasing polypeptide. It is a hormone belonging to the glucagon-secretin family and showing similarities of structure with secretin, glucagon and GLP-1(7-36)amide (Schmidt et al., 1985).
1.3.1 A Historical Perspective of GIP

GIP was discovered by Brown & Pederson in 1970 whilst investigating the effect of a porcine cholecystokinin-pancreozymin (CCK) preparation (prepared by Jorpes & Mutt, 1961) on gastric acid secretion in dogs. They observed that a 40% pure CCK preparation stimulated acid secretion more strongly than a 10% pure CCK preparation. From this it was hypothesized that either a stimulator of gastric acid secretion had been selectively concentrated, or a second inhibitory hormone was present in the 10% preparation which was removed on purification. Brown and co-workers had previously published that they could isolate, from a side fraction of the CCK purification, a polypeptide which was inhibitory for H⁺ secretion, pepsin secretion and motor activity in the antral and fundic pouches of dogs (Brown et al., 1969). Sequence determination of the peptide showed an amino acid composition distinct from any known gastrointestinal peptide and so it was given the name gastric inhibitory polypeptide (Brown et al., 1970).

With the purification of the peptide components of the 10% CCK preparation it became possible to study their effects on immunoreactive-insulin (IRI) levels. Studies in man and rats showed that 10% CCK enhanced IRI concentration in blood when glucose was simultaneously administered. Highly purified CCK at a corresponding dose rate failed to reproduce the insulinotropic effect (Brown et al., 1975). Subsequent infusion of porcine GIP with glucose demonstrated that the potentiating effect of 10% CCK on IRI rise, during an intravenous glucose infusion, is largely attributable to GIP.
1.3.2 Isolation and Purification of GIP

Porcine GIP, as isolated and purified by Brown and colleagues (1970) was shown to be homogeneous by polyacrylamide gel electrophoresis. It was initially characterised as a straight chain peptide of 43 amino acids with a calculated molecular weight of 5105 daltons (Brown & Dryburgh, 1971). It has since been demonstrated that this sequence determination was wrong by an extra glutamine residue after position 29. Porcine GIP is now known to consist of 42 amino acid residues (Jörnvall et al., 1981).

Subsequently, both the bovine and human forms of GIP have been sequenced. Both have been shown to contain 42 amino acids. Species variations in amino acid content have been found. Porcine and human GIP differ at residues 18 and 34 (Moody et al., 1984; Carlquist, 1987). Porcine and bovine GIP's differ at residue 37 (Carlquist, 1987). The physiological importance of these species variations remains to be established. No natural truncated forms of GIP have been found but a synthetic replicate of human GIP\textsubscript{1-37} retains the biological activity of natural human GIP (Carlquist, 1987).

1.3.3 Distribution of GIP within the Gastrointestinal Tract

The cellular location and hence distribution of a hormone is established by immunolocalisation methods. GIP was first suggested to be located in the D\textsubscript{1} cell of the duodenum and jejunum in dogs and man (Polak et al., 1973). Subsequent studies in man, pigs and dogs indicated that immunofluorescent GIP cells were ultrastructurally indistinguishable from the K cells (Buffa et al., 1975). The K cell was first identified histochemically in 1975, by Solcia and co-workers (Solcia et al., 1975). The D\textsubscript{1} cell is not involved in GIP production. In mammals GIP-secreting K cells are located exclusively in the small intestine, being concentrated mainly in the duodenum and upper jejunum of man (Bloom & Polak, 1981). In the rat and mouse, however, the K cells
are as numerous in the ileum as in the upper small intestine (Solcia et al., 1989). In man, GIP release studies in response to glucose stimulation have confirmed the proximal small intestine to be the major, but not exclusive site of endogenous GIP release. Small quantities of GIP also being released by the distal small intestine (Thomas et al., 1977). This compares favourably with the distribution of GIP reported by the extraction studies on human tissue by Bloom & Polak (1981).

1.3.4 The Molecular Forms of Immunoreactive GIP

Immunoreactive GIP (IR-GIP) as detected by RIA can be separated on a molecular weight basis by gel filtration. In man, gel filtration of plasma or serum demonstrates three distinct peaks of immunoreactivity; one of high molecular weight which elutes in the void volume ($V_0$) of the column, one of a molecular weight of approximately 8000 daltons and one of approximately 5000 daltons. The 5000 dalton peak corresponds with the elution position of synthetic porcine GIP (Brown et al., 1979). Gel filtration of gastrointestinal mucosal extracts of porcine and human tissue fails to detect the $V_0$IR-GIP but does detect a fourth peak of immunoreactivity eluting between the 8000 dalton peak and the void volume. This fourth peak has been suggested to be a prohormone of 5000 IR-GIP (Krarup, 1988).

It has been shown that pre-treatment of serum (boiling or addition of concentrated urea) reduces the amount of $V_0$IR-GIP and increases 5000 IR-GIP. On the basis of this it has been suggested that $V_0$IR-GIP represents non-specific binding of 5000 IR-GIP to large molecular weight serum proteins (Krarup, 1988; Jones et al., 1987). $V_0$IR-GIP may however represent interference of serum proteins alone as reactivity of antisera to 5000 IR-GIP is not paralleled by reactivity to $V_0$IR-GIP. GIP itself, therefore, may not be involved in this non-specific binding (Krarup, 1988). The levels of $V_0$IR-GIP are unaffected by ingestion of test meals, glucose or fat (Krarup et al., 1985; Krarup et al., 1987a). $V_0$IR-GIP is a problem in assays as it has been
shown to vary substantially between plasma samples (Krarup et al., 1985). Sensitivity of the assay is therefore important to reduce the amount of plasma sample in the assay and thereby reduce the interference of V₀IR-GIP.

The 8000 IR-GIP is not believed to be a prohormone. Extraction and characterisation of this immunoreactive form from the porcine small intestine, has shown that it does not contain the complete amino acid sequence of 5000 IR-GIP (Otte et al., 1984). Pancreas perfusion studies have shown the 8000 IR-GIP not to be insulinotropic in nature (Krarup et al., 1987b). Following oral stimulation, 8000 IR-GIP has been reported to increase in one study (Krarup et al., 1987a), while previous work by the same group reported a very modest increase in 8000 IR-GIP after a meal with inconsistencies in the response detected by different antisera (Krarup et al., 1985).

A number of different antisera have now been successfully raised against porcine GIP (Kuzio et al., 1974; Morgan et al., 1978; Sarson et al., 1980a). Unfortunately, whilst all the antisera recognise the three molecular components of plasma GIP, they differ considerably in their recognition of the relative amounts of the various forms present. All the antisera do, however, recognise the 5000 IR-GIP to be the major form. The cross reactivity of human plasma GIP with the various antisera available has been reviewed by Jorde et al. (1983). Characteristics of the various antisera have been reviewed by Krarup (1988). Differences in cross reactivity of porcine antisera with human GIP are due to the different binding regions available in the antisera. Most antisera raised against porcine GIP have binding regions between residues 15 to 42. Unfortunately this is the region of species variations in amino acid sequence. Whilst there is general agreement in the literature about GIP release patterns in response to the ingestion of nutrients in man, the absolute values measured differ according to the antiserum used (Jorde et al., 1983). One group have reported fasting values five times lower than those reported by other groups (Sarson et al., 1980a). This has led to some confusion in the early work on plasma GIP concentrations in
clinical situations. The characteristics of the antiserum raised at Surrey are discussed fully in the Methods Chapter.

1.3.5 **Stimuli for GIP Release from the Endocrine K Cell**

For GIP to be considered a peptide of the EIA it is essential that it is released into the circulation by the ingestion of secretagogues known to release insulin (Brown, 1982). Intestinal perfusion studies in rats have shown that GIP is stimulated by monosaccharides which are actively transported by the sodium dependent hexose pathway (Sykes et al., 1980). The use of phloridzin, a β-D-glucoside, has confirmed that absorption of the sugar is necessary for GIP secretion, not just the presence of the sugar in the intestinal lumen (Sykes et al., 1980; Creutzfeldt & Ebert, 1977). Studies, in man and rats, with sugars which do not meet the structural requirements for active transport (an intact pyranose ring, a hydroxyl group at C6 and a hydroxyl group in the alpha position on C2) fail to stimulate GIP secretion (Sykes et al., 1980; Salminen et al., 1982).

For mice, however, the situation is not so clear. Fructose stimulates GIP release in the obese hyperglycaemic mutant (ob/ob) mice (Flatt et al., 1989). This may be explained by the metabolism of fructose (fructolysis) into glucose and lactate within the intestinal epithelial cell of these animals, or possibly the sodium-dependent glucose transporter is not an exclusive stimulus for GIP secretion in these animals.

On a calorigenic basis, fat, in the form of triglycerides, is a more potent stimulator of GIP release than glucose (Jorde et al., 1983; O'Dorisio et al., 1976; Brown et al., 1975; Falko et al., 1975). In contrast free fatty acids, mono and diglycerides have demonstrated variable GIP responses (Ross & Shaffer, 1981; Williams et al., 1981). The mechanism by which triglycerides stimulate GIP is unknown, but for individual fatty acids it has been shown in mice that an increased
chain length and a greater degree of unsaturation enhance GIP release (Kwasowski et al., 1985). It has been suggested that differences in intracellular metabolism of fat may account for the different GIP responses (Kwasowski et al., 1985). As with sugars, absorption of fat into the epithelial cell is a necessary prerequisite for GIP release. In man, fat malabsorption syndrome due to chronic pancreatitis produces an impaired GIP response to an oral fat load. This can be partially restored by the provision of digestive pancreatic enzymes with the meal (Ebert & Creutzfeldt, 1980a). Coeliac patients have also been shown to have very poor GIP responses to a test meal, even though the number of immunofluorescent GIP cells in their small intestine was normal (Creutzfeldt et al., 1976).

Intraduodenal administration, in man, of a mixture of amino acids (namely arginine, histidine, isoleucine, lysine and threonine) has been reported to produce a marked rise in GIP (Thomas et al., 1978). In contrast, in mice, individual amino acids produce modest and transient GIP increases with no difference in potency between the amino acids tested (Flatt et al., 1991). Protein is generally absorbed in man as di- and tripeptides by a sodium-dependent mechanism (Munck, 1981). Hence, the luminal concentration of amino acids produced by Thomas and co-workers in their study was far greater than that normally seen. Use of whole proteins (280g fillet steak or 45g of a meat extract) has failed to produce a significant elevation in serum GIP levels (Cleator & Gourley, 1975). The influence of di- and tripeptides on GIP secretion has yet to be investigated.

On an individual nutrient basis, fats and carbohydrates are the major stimuli for GIP secretion. Nutrients however are rarely ingested alone but more usually in a complex mixed meal form. The interaction of nutrient stimulatory effects must also be considered. It has been suggested that amino acids may modify the GIP secretory response to a meal; corn oil-stimulated GIP release in dogs can be progressively inhibited by increasing doses of the amino acids alanine or arginine (Williams et al.,
The physical form of a meal can also influence the postprandial GIP response. If volume is standardised then the size of an oral glucose load correlates with postprandial plasma GIP levels in man (Hampton et al., 1986). Altering the composition of a solid meal (200-1200 kcal/meal), whilst maintaining its size also shows a linear relationship with postprandial GIP response (Service et al., 1983). Physical form, volume and energy content can all interact to alter gastric emptying (Low, 1991) and thus alter the GIP response.

1.3.6 The Evidence for GIP as a Incretin

The evidence for an insulinotropc role for GIP is inferred from studies where porcine GIP was infused into man and animals, and from the addition of porcine GIP to cell cultures. Many workers in the earlier studies failed to provide information on the purity of the hormone preparation used and the prevailing glucose concentration. In many of these studies the doses of GIP used were supraphysiological and the hyperglycaemic environment was in excess of normal postprandial increases (Dupré et al., 1973; Elahi et al., 1979). In 1984, the physiological importance of GIP was still in doubt (Sarson et al., 1984). More recent studies support the opinion of GIP as an insulin secretagogue in humans (Jones et al., 1987; Jones et al., 1989a; Kreymann et al., 1987; Rasmussen et al., 1990) and in animals (Flatt et al., 1984). Human synthetic GIP has been shown to potentiate insulin secretion at physiological concentrations (Kreymann et al., 1987) and to be more potent than porcine GIP (Füessl et al., 1988; Füessl et al., 1990).

Immunoneutralisation of endogenous GIP indicates that for rats about 20% of the augmentation seen after the administration of intraduodenal compared with intravenous glucose can be attributed to GIP (Ebert & Creutzfeldt, 1982; Ebert et al., 1983). When animals are unrestrained and not anaesthetised GIP antiserum greatly diminishes the incretin effect (Lauritsen et al., 1981). This discrepancy between the
studies is most probably due to differences in their methodology and the contribution in
the neural component of the EIA.

1.3.7 Stimulation of Insulin Secretion by GIP

As mentioned in previous sections of this Chapter, GIP only stimulates insulin
secretion under mild to moderate hyperglycaemic conditions. In rats, the degree of
glycaemia required has been assessed as a plasma glucose concentration greater than
5.5mM (Anderson et al., 1978). Glucose, in modulating the response of the B cells to
the presence of GIP protects the organism from inappropriate hyperinsulinaemia
leading to hypoglycaemia.

For GIP to stimulate the B cells, receptors must be present capable of binding
GIP. Evaluation of GIP receptors is difficult for two major reasons; the iodination
methods used for GIP and the methods for isolating islets. Iodination of GIP produces
a heterogeneous population of iodinated peptides and also changes in the GIP molecule
resulting from the fact that iodination alters biological activity (Brown et al., 1982).
However in 1976, it was reported that two hydrophobic amino acid areas in peptides of
the glucagon family are probably important for receptor binding (Blundell et al.,
1976). In the GIP molecule the sequence regions 6-14, and 19-27, are the
hydrophobic regions. Modification of Tyr\(^{10}\) by iodination and Met\(^{14}\) and Tyr\(^{25}\) by
oxidation can therefore be expected to interfere with receptor binding.

Cell isolation methods currently use collagenase to digest away extraneous
material. Yet there is evidence that collagenase critically influences the GIP receptor
(Brown, 1982). Some success has been obtained with hamster transplantable
insulinoma cells because of the relatively high density of the binding sites. Studies
have found two types of binding site: a small population of high affinity sites, and a
larger population of low affinity sites (Amiranoff et al., 1985). Binding of GIP to its
receptor was found to be specific, saturable and reversible. Glucagon did not alter binding of $^{125}$I-GIP to pancreatic B cells.

Glucose is the main physiological stimulator of insulin secretion being metabolised by the B cells and so enhancing adenosine triphosphate (ATP) production (reviewed by Hedeshkov, 1980; Berggren et al., 1992). Sharp in 1979, reported that raising the cyclic adenosine mono-phosphate (cAMP) concentration of the B cell enhanced the effects of glucose on insulin stimulation. Other workers have since elucidated that GIP increases cAMP accumulation in the islets (Szecowka et al., 1982). GIP achieves this by activating adenylate cyclase in the plasma membrane, which is responsible for the conversion of ATP to cAMP (Flatt & Bailey, 1991). The elevation of cAMP by GIP is insufficient to stimulate insulin secretion on its own, simultaneous glucose metabolism generating ATP is necessary for increased insulin secretion. In a recent study GIP was also suggested to activate another plasma membrane associated enzyme, phospholipase A$_2$ (PLA$_2$) (Lardinois et al., 1990). This has not been studied by other workers.

Early studies suggested that GIP did not stimulate glucagon secretion from the A cells of the pancreas at physiological concentrations (Brown et al., 1975; Pederson & Brown, 1978; Elahi et al., 1979). More recently GIP has been shown to enhance glucagon secretion under basal conditions (Kreymann et al., 1987; Kreymann & Bloom, 1991). Glucagon itself is known to stimulate insulin release in the same manner as GIP, activating adenylate cyclase and thus promoting an increase in cAMP (Schuit & Pipeleers, 1986; Berggren et al., 1992). Glucagon and GIP therefore have a synergistic effect on glucose-induced insulin release (Berggren et al., 1992).
1.3.8  Inhibition of GIP Release from the K Cell

Although there is no generally accepted mechanism, a negative feedback situation has been proposed. For a negative feedback role to be considered the substance, hormone or metabolite, must be liberated by GIP in response to nutrient absorption. It has been shown in man that the magnitude of the GIP response to fat absorption can be reduced by a simultaneous intravenous glucose load (Cleator & Gourley, 1975; Creutzfeldt et al., 1976; Crocket et al., 1976; Verdonk et al., 1980). A negative feedback control of insulin on fat stimulated GIP in normal subjects was first suggested by Brown et al. (1975). This feedback was clearly demonstrated by the infusion of exogenous insulin simultaneously with an oral fat load. Negative feedback on fat-stimulated GIP secretion has also been verified by Creutzfeldt et al. (1980) in patients with no endogenous insulin secretion i.e. insulin dependent diabetics. Creutzfeldt demonstrated in these patients that glucose-induced GIP secretion was not reduced by exogenous insulin infusion. In obese subjects however, exogenous insulin fails to reduce fat stimulated IR-GIP release (Creutzfeldt et al., 1978).

The effects of glycaemia on GIP secretion have also been investigated (Verdonk et al., 1980) but it is difficult to isolate the effects of glycaemia from the effects of hyperinsulinaemia. Creutzfeldt et al. (1980) reported a slight suppressive effect of hyperglycaemia on fat-induced GIP secretion in the absence of insulin. Prolonged hyperinsulinaemia, possibly induced by glucose clamping, has been shown to reduce the effect of insulin on the K cell (Stockman et al., 1984) possibly by reducing the sensitivity of the K cell to insulin.

The importance of a feedback system is acknowledged in order to protect the organism from inappropriate hyperglycaemia. If the K cells respond to the circulating insulin concentration, the question remains how the cell can distinguish fat stimulation from glucose stimulation. It has been suggested that the K cell does not respond to
insulin concentration directly but indirectly to the increased utilisation of glucose
induced by insulin (Creutzfeldt et al., 1980).

The importance of feedback on fat simulation of GIP for species that naturally
consume a low fat diet is debatable and species differences in feedback mechanisms
may exist. In contrast to the observations in man, a reduced fat attenuation of the GIP
response to an intraduodenal glucose load has been reported in dogs (Sirinek et al.,
1978). One study has even suggested a role for C-peptide II in a feedback mechanism
in rats (Dryburgh et al., 1980). To date no other workers have studied this.

1.3.9 Other Metabolic Effects of GIP

GIP has been shown to act synergistically with insulin to augment insulin
dependent inhibition of liver glycogenolysis in both rodents and man (Hartmann et al.,
1986; Elati et al., 1986). In addition to its interactions with insulin, GIP also
demonstrates independent direct physiological actions on adipose tissue metabolism.
GIP possesses a strong antilipolytic action on glucagon stimulated lipolysis by
selectively and competitively blocking the activation of adenylate cyclase by glucagon
(Ebert & Brown, 1976). Activation of adipose tissue lipoprotein lipase in 3T3-LI cells,
a mouse fibroblast cell line which resembles an adipocyte, by GIP (Eckel et al., 1978)
is potentially an important mechanism for the clearance of triglycerides from
chylomicrons after feeding. GIP has also been shown to stimulate fatty acid synthesis
in certain deposits of rat adipose tissue (Oben et al., 1991c). All of these actions
complement the incretin role of GIP.
1.4 GLP-1(7-36)amide

Glucagon-like peptide-1 (GLP-1) a hormone from the same family as GIP is located in the small and large intestine. Two truncated forms of the hormone have been identified, GLP-1(7-37) and GLP-1(7-36)amide. Both truncated forms are equipotent insulinotropic hormones in the perfused rat pancreas (Weir et al., 1989; Suzuki et al., 1989). Additionally it has been suggested that GLP-1 and GLP-2 (see Figure 1.1 and Section 1.4.2 and 1.4.3) may be neurotransmitters as both have been found to activate rat brain adenylate cyclase (Hoosein & Gurd, 1984). However, it is their potential involvement as incretins of the EIA which is reported here.

1.4.1 A Historical Perspective of Gut Glucagon-Like Peptides

In 1961, Unger and coworkers demonstrated that material present in extracts of canine and human intestine could cross react with antibodies directed towards the N terminal/central regions of the pancreatic glucagon molecule. This material failed to bind to antibodies directed towards the C terminal part of pancreatic glucagon (Unger et al., 1961; Moody, 1980). The term glucagon-like immunoreactivity (GLI) was proposed by Unger, to distinguish this material from pancreatic glucagon. Gut glucagon-like immunoreactivity was subsequently shown to be present in extracts of intestinal tissue from rats, dogs and humans (Unger, 1961; Unger, 1966; Samols et al., 1966; Unger, 1968).

Gut glucagon-like immunoreactivity was shown to be heterogeneous in nature (Samols et al., 1966). A classification system was developed on the basis of approximate molecular weight and reactivity of the hormone towards various glucagon antisera. It is now known that pancreatic glucagon and intestinal glucagon are products of tissue specific processing of the proglucagon hormone. A simpler nomenclature
system, based on proglucagon residues, has therefore been established (reviewed by Conlon, 1988).

1.4.2 **Isolation of GLP-1 (Proglucagon 72-108)**

Molecular biological advances during the 1980's, with the production of complementary DNA (cDNA) to messenger RNA (mRNA) for proglucagon, enabled the elucidation of the complete amino acid sequence of mammalian proglucagon. Lund and co-workers were the first to report that the preproglucagon gene from angler fish as well as encoding glucagon encodes another peptide with a high degree of homology with glucagon, *glucagon related COOH peptide* (Lund et al., 1982). Their findings were confirmed by Bell and co-workers using a cDNA raised against hamster mRNA, they found not one but two peptides with an amino acid structure similar to glucagon, *glucagon-like peptide-1* and *glucagon-like peptide-2* (Bell et al., 1983b). It was later determined that preproglucagon mRNA in the pancreas, ileum and colon are identical and that identical transcription start sites are used in all three tissues (Mojsov et al., 1986; Conlon, 1988). The diversity of peptides found in the gut and pancreas is therefore concluded to be due to tissue-specific post-translational processing (George et al., 1985; Mojsov et al., 1986; Ørskov et al., 1986). Tissue specific processing of preproglucagon has been reviewed by Conlon (1988) and Drucker (1990). It is illustrated in Figure 2. Not all researchers in this area recognise such distinct tissue specific processing. Mojsov and coworkers report that the pancreas does contain some free GLP-1 (in the forms GLP-1(7-37) and GLP-1(7-36)amide) though the concentrations are far lower than those seen in the gut (Mojsov et al., 1986).
Figure 1.1  
**Tissue Specific Processing of Preproglucagon in the Pancreas and Gastrointestinal Tract**

![Diagram of tissue-specific processing of Preproglucagon](image)

Modified from Conlon (1988).

The coexistence of glicentin, GLP-1 and GLP-2 immunoreactivity within a single enteroglucagon cell secretory granule was reported by Vardell *et al.* (1985). The L cell of the intestinal mucosa was reported to be the cellular localisation of enteroglucagon in 1976. (Moody *et al.*, 1976). This was subsequently confirmed in the rat, dog and cat by Larsson & Moody (1981).
1.4.3 Isolation and Sequence Determination of GLP-1(7-36)amide

GLP-1 (proglucagon 72-108) is a peptide of 37 amino acid residues. It is cleaved at residue 6, a single basic residue, to produce GLP-1(7-37). The C terminal glycine residue would be expected to give up its amino grouping to amidate the terminal arginine residue (Bradbury et al., 1982) producing GLP-1(7-36)amide. The truncated form of the peptide GLP-1(7-36)amide has been shown to be the major molecular form in intestinal tissue from rats, pigs and humans (Kreymann et al., 1987; Kreymann et al., 1988; Ørskov et al., 1989). Perfused pancreas experiments in rats have enabled identification of the important amino acid residues for insulinotropic activity. Amidation of the C terminal residue had no significant effect on activity of the hormone in this capacity (Suzuki et al., 1989). Histidine at position 7 as a free terminal amino acid is important for insulinotropic activity (Suzuki et al., 1989).

GLP-1 (prohormone 72-108) is an important peptide being found in all proglucagons isolated so far. The amino acid sequence is highly conserved across species being identical in rat (Heinrich et al., 1984) hamster (Bell et al., 1983b) bovine (Lopez et al., 1983) and human (Bell et al., 1983a) preproglucagons. GLP-1(7-36)amide and GLP-1(7-37) also show a strong degree of homology at the amino terminal end of the molecule to glucagon. Fourteen out of twenty-nine comparable residues are the same (Varndell et al., 1985).

The importance of GLP-2 is as yet unknown. No physiological role has yet been found and it is absent in some non-mammalian preproglucagons (Lund et al., 1982).
1.4.4 Distribution of GLP-1(7-36)amide within the Gastrointestinal Tract

As mentioned previously in this Chapter, gut glucagon-like immunoreactivity is located in the L cell of the intestinal mucosa. Extraction of intestines and determination of the distribution of a hormone is dependent on accurate identification of the intestinal regions. Within the small intestine, the distinction between duodenum, jejunum and ileum is important to make, yet few researchers accurately report where they determined the different regions to be. Extraction of intestines and determination of the distribution of GLP-1(7-36)amide in rats reveals that the highest concentrations are located in the terminal ileum and colon in both normal and diabetic animals (Kreymann et al., 1988). This report compares well with the distribution of gut glucagon-like immunoreactivity reported by earlier workers. Low concentrations of glucagon-like immunoreactivity have been found in the duodenum and jejunum, with tissue concentrations rising progressively to a maximum in the lower ileum and colon in humans (Bloom & Polak, 1978), primates (Bryant & Bloom, 1979), pigs (Holst et al., 1987) and the rat (Perez-Castillo & Blazques, 1980). In contrast, oxyntomodulin (proglucagon 33-69) another product of the L cell, has been shown to be more widely distributed throughout the intestine. Significant amounts of oxyntomodulin were found in the jejunum and caecum as well as the ileum and colon (Kervran et al., 1987). This discrepancy is almost certainly due to differences in identification and reporting of the areas of the small intestine.

1.4.5 The Molecular Forms of Immunoreactive GLP-1 (Prohormone 72-108)

Gel filtration of porcine plasma for basal IR-GLP-1(7-36)amide, and postprandial IR-GLP-1(7-36)amide, demonstrates that most of the IR-GLP-1 measurable in plasma in the basal state is inactive. This is due to cross reaction of the antiserum with the inactive bound pancreatic form, pancreatic C terminal residue. Nutrient stimulation causes an increase in active, intestinal GLP-1(7-36)amide with a corresponding decrease in the pancreatic form (Orskov et al., 1987a). As a
consequence of this cross-reactivity of the antiserum, the apparent changes in circulating levels of IR-GLP(7-36)amide are small (Ørskov et al., 1987a). This has created problems with assay sensitivity and specificity. Very few research groups have successfully raised antisera to GLP-1(7-36)amide. Those that have, have found that their antisera cross-react with the parent GLP-1 molecule (prohormone 72-108) (Kreymann et al., 1987; George et al., 1985; Uttenthal et al., 1985; Ørskov et al., 1987a). Some antisera also cross-react with proglucagon C terminal residue peptide released from the pancreas (Ørskov et al., 1987b).

1.4.6 Stimuli for GLP-1(7-36)amide Release from the Entero-Endocrine L-Cell

GLP-1(7-36)amide immunoreactivity has been shown to be released into the circulation following a mixed meal or in response to an oral glucose load (Kreymann et al., 1987). Peak IR-GLP-1(7-36)amide response occurred 30 minutes after the administration of the test meal.

The mechanism of GLP-1(7-36)amide secretion from the L cell has only recently received attention. One group of workers have published results from ileal loop perfusion studies in dogs (Shima et al., 1990). They investigated sugar stimulation of GLP-1(7-36)amide secretion and concluded that only actively absorbed sugars stimulate GLP-1(7-36)amide release. This confirmed previous observations of other workers that D-glucose is a stimulus for GLP-1 secretion in porcine ileum (Ørskov et al., 1986) and in man (Kreymann et al., 1987). The study by Shima and co-workers was interesting as they reported that absorption and subsequent metabolism of the sugar was not responsible for GLP-1(7-36)amide release. The sugar had to meet specific stearic requirements to stimulate GLP-1(7-36)amide release. Disaccharides also stimulated GLP-1(7-36)amide release, but interestingly their effects appeared to be direct acting and not via their hydrolysis products.
These results require confirmation but initially this seems a strange situation of hormone release in response to a nutrient stimulus which is still to all extents external to the body. Activation of a hormonal pathway for the stimulation of insulin secretion under these circumstances could well be dangerous.

1.4.7 The Evidence for GLP-1(7-36)amide as an Incretin

Initial investigators examining the incretin effect used full-length GLP-1 (proglucagon 72-108). This was shown to have only a weak stimulatory activity upon insulin release in isolated islets (Schmidt et al., 1985), and in ob/ob mice (Bailey & Flatt, 1987). Truncated forms of GLP-1 (both GLP-1(7-37) and GLP-1(7-36)amide) are considered physiologically important incretins based on evidence from cultured islet experiments (Drucker et al., 1987; Flatt et al., 1990) and pancreas perfusion studies (Shima et al., 1988). Infusion studies in humans at physiological post-prandial concentrations have shown GLP-1(7-36)amide to be the most potent enhancer of insulin secretion yet identified (Kreymann et al., 1987).

Pancreas perfusion studies have demonstrated that truncated GLP-1 shows glucose dependency for its action on insulin secretion (Shima et al., 1988).

1.4.8 Stimulation of Insulin Secretion by GLP-1(7-36)amide

As with the studies looking at B cell receptors for GIP, insulinoma cells have been utilised to investigate potential receptors and their characteristics for truncated GLP-1. Specific receptors have been found on rat insulinoma cells. Binding of GLP-1(7-36)amide has been found to be saturable and time dependent, (Ørskov & Nielsen, 1988; Göke & Conlon, 1988). These receptors were highly specific being completely separate from glucagon receptors. Glucagon and GIP failed to influence the binding of GLP-1(7-36)amide. Receptors have also been reported in rat brain and lung membranes (Kanse et al., 1988).
Binding of GLP-1(7-36)amide to its receptor has been shown to increase cAMP concentration in a dose dependent manner via its activation of adenylate cyclase (Göke & Conlon, 1988; Göke et al., 1989; Drucker et al., 1987). GLP-1(7-36)amide therefore stimulates insulin in the same manner as GIP and glucagon. Unlike GIP, GLP-1(7-36)amide suppresses glucagon secretion (Kreymann et al., 1987; Fridolf et al., 1990a; Fridolf et al., 1990b; Yamato et al., 1990). This offers another method by which truncated GLP-1 can control postprandial glucose levels. Full length GLP-1 can bind to the truncated GLP-1 receptor but it is 200 times less potent than its truncated form (Göke & Conlon, 1988).

1.4.9 Other Metabolic Effects of GLP-1(7-36)amide

GLP-1(7-36)amide has been shown to directly stimulate fatty acid incorporation and fatty acid synthesis in adipose tissue (Oben et al., 1991c).

1.5 Comparison of GIP and GLP-1(7-36)amide

GIP and GLP-1(7-36)amide share 13 amino acid residues in common. They each show glucose dependency for their incretin effect and both stimulate insulin release by the adenylate cyclase system and cAMP synthesis. GLP-1(7-36)amide is on a molar basis four times as potent as GIP at stimulating insulin release, but it's circulating levels are much lower both in the basal and postprandial states (Kreymann et al., 1987). Both hormones also have direct metabolic actions on adipose tissue.

GIP and GLP-1(7-36)amide can interact with each other in a synergistic manner to stimulate cAMP (Fehmann et al., 1990a). GIP and GLP-1(7-36)amide can also interact with CCK-8, one of a variety of truncated forms of cholecystokinin (CCK) (Fehmann et al., 1990b; Zawalich, 1988). CCK-8 is a neural component of the EIA; it has the ability to augment insulin secretion in response to amino acid stimulation.
CCK-8 stimulates insulin secretion by means of an alternative secondary messenger system to that used by GIP and GLP-1(7-36)amide. CCK interacts with phospholipase C in the plasma membrane with the generation of diacylglycerol and inositol (1,4,5) triphosphate (Flatt & Bailey, 1991; Berggren et al., 1992).

1.6 The Role of the EIA in Disease States

Manipulation of the habitual diet has been demonstrated to modify basal and acute nutrient stimulated GIP secretion in rats (Hampton et al., 1983) and in humans (Ebert et al., 1979). GLP-1(7-36)amide levels have been reported to be increased in the large intestine in hyperphagic, streptozotocin diabetic rats (Kreymann et al., 1988).

Circulating gut hormone levels are therefore of interest in disease states which are known to have a strong dietary link. The vast majority of studies have concentrated on IR-GIP levels due to its earlier discovery. Investigations into GLP-1(7-36)amide levels are complicated by the lower circulating concentration of this hormone and its fragility making assay sensitivity a problem. Gut glucagon-like immunoreactivity, as measured with N-terminal directed glucagon antisera, has been shown to be significantly elevated in coeliac disease (Bloom & Polak, 1981) and in obese patients following jejuno-ileal by-pass surgery (Sarson et al., 1980b). GLP-1(7-36)amide levels have been shown to be elevated in patients with postgastrectomy dumping syndrome (Kreymann et al., 1987).
1.6.1 Clinical Studies

As yet no pathological condition has been found which is causally related to hyper- or hyposecretion of GIP. Changes in plasma circulating IR-GIP concentrations have however been described for many disease states (Sarson & Bloom, 1981). Table 1.2, provides an outline of clinical studies concerned with changes in plasma GIP levels in insulin-dependent diabetes mellitus (IDDM), non-insulin dependent diabetes mellitus (NIDDM) and obesity. These three diseases have attracted considerable attention in this area, a correlation between GIP concentrations and insulin levels has so far not been proven. Interpretation of the results of the clinical studies is not aided by: the wide variation in study protocol used, the number of subjects, the provision of adequate controls, the nature of the provocative test, consideration of the influence of previous dietary habits and medicinal treatment of subjects. In addition to protocol differences, assays were performed using different RIA methods. The cross-reactivity of the porcine antisera to human GIP will also have influenced the results. Recent studies have attempted to control the possible variations in individual response by obtaining homogeneous subject groups and standardising the method used.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Nature of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willms et al.</td>
<td>1978</td>
<td>Increased basal GIP.</td>
</tr>
<tr>
<td>Reynolds et al.</td>
<td>1979</td>
<td>11 Patients with IDDM treated with insulin. Last insulin dose 4 hours before test. Basal GIP levels normal. Reduced GIP response to a 1g/kg body weight glucose load.</td>
</tr>
<tr>
<td>Creutzfeldt et al.</td>
<td>1980</td>
<td>40 patients insulin-treated for ≥ 5 years. Body weight within 10% ideal. Insulin dose withheld night before test and CHO content of meal reduced by 50%. Increased basal GIP levels and increased response to glucose load (100g glucose equivalent). Test repeated with simultaneous insulin infusion; again similar GIP response. Fat tolerance test (150ml suspension corn oil) with iv glucose infusion gave similar increased GIP response.</td>
</tr>
<tr>
<td>Krarup et al.</td>
<td>1983</td>
<td>15 patients. GIP response to standard meal (490 Calories; 60% CHO, 26% fat, 14% protein) measured 7 days, 14 days and 3 months after diagnosis. Basal GIP levels normal on all 3 occasions and GIP response to the meal was reduced at all 3 time points.</td>
</tr>
<tr>
<td>Service et al.</td>
<td>1984</td>
<td>5 patients. Normal basal GIP levels and normal response to a standard meal (6 Cal/kg ideal body weight, 50% CHO, 30% fat, 20% protein).</td>
</tr>
<tr>
<td>Krarup et al.</td>
<td>1985</td>
<td>15 subjects. GIP response to standard meal examined 7 days, 14 days and 3, 6, 9, 12 and 18 months after diagnosis. Standard meal same as in previous study. GIP response to the meal reduced on the first 4 occasions. GIP response subsequently normalised as B cell function declined. 9 months after diagnosis no significant difference was found from GIP response of normal subjects. Intensive insulin treatment at the point of diagnosis caused a faster normalisation of GIP levels.</td>
</tr>
</tbody>
</table>
### Table 1.2b  
**Literature Review of Clinical Studies Investigating the Potential Involvement of GIP in Non-Insulin Dependent Diabetes Mellitus**

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Nature of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown et al.</td>
<td>1975</td>
<td>9 patients within 20% ideal body weight, treated by diet alone. Some previously treated by oral hypoglycaemic agents, discontinued 3 days before test. Basal GIP levels normal. In response to an oral glucose tolerance (50g) the GIP response was exaggerated. In response to a 66g triglyceride load incremental GIP response was greater but the integrated response was similar to the controls.</td>
</tr>
<tr>
<td>Ross et al.</td>
<td>1977</td>
<td>20 patients non obese, treated by diet alone (previously treated by use of oral hypoglycaemic agents stopped 1 week before test). Basal GIP levels normal, with an exaggerated GIP response to a 50g glucose load.</td>
</tr>
<tr>
<td>Ebert et al.</td>
<td>1980b</td>
<td>141 patients - GIP response to a test meal was normal for 18%, diminished for 27% and exaggerated for 55% of patients. GIP response to an oral glucose load (100g) was normal for 12%, diminished for 34% and exaggerated for 54% of patients.</td>
</tr>
<tr>
<td>Levitt et al.</td>
<td>1980</td>
<td>12 patients - 6 with clinical evidence of autonomic neuropathy (AN) treated by diet and oral hypoglycaemic agents. Basal GIP levels normal. In response to a mixed meal (36.4g fat, 72g CHO and 43g protein) those patients without AN gave a normal GIP response. Patients with AN showed a significantly reduced GIP response at all time points.</td>
</tr>
<tr>
<td>Salera et al.</td>
<td>1982</td>
<td>14 patients (non obese) 15 patients (obese). Non obese patients had normal basal and acute stimulated GIP levels in response to 75g of glucose. Obese patients had normal basal GIP levels with increased response to 75g glucose.</td>
</tr>
<tr>
<td>Elahi et al.</td>
<td>1984</td>
<td>10 patients, none had received insulin or oral hypoglycaemic agents. Hyperglycaemic clamp procedure used. Basal GIP levels markedly elevated. Glucose tolerance test (40g/m² body surface) incremental GIP response smaller than with control patients.</td>
</tr>
<tr>
<td>Service et al.</td>
<td>1984</td>
<td>5 normal weight and 5 obese patients. Normal basal GIP levels. Reduced GIP response to standard meal (50% CHO, 30% fat and 20% protein). Reduced mean GIP response was due entirely to a reduced response in obese patients.</td>
</tr>
<tr>
<td>Mazzaferri et al.</td>
<td>1985</td>
<td>19 Caucasian diabetics (lean and obese), 11 American Indian diabetics (obese). High fasting GIP levels and exaggerated GIP responses to nutrients seen. American indians had consistently greater GIP responses to tests.</td>
</tr>
<tr>
<td>Study</td>
<td>Year</td>
<td>Methodology</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Osei et al.</td>
<td>1986</td>
<td>5 patients, poorly controlled. Basal GIP levels normal. In response to a test breakfast (500kCal), a significantly increased GIP level at 20 minutes post prandially.</td>
</tr>
<tr>
<td>Nauck et al.</td>
<td>1986</td>
<td>14 patients, lean and moderately obese. Oral hypoglycaemics (taken by 5 patients) stopped 3 days before test. Oral glucose load (50g glucose + low molecular weight oligomers) and isoglycaemic iv glucose infusion performed. Mean basal GIP values and GIP responses to oral glucose were normal. The range of GIP responses was however wider in patients than controls.</td>
</tr>
<tr>
<td>Jones et al.</td>
<td>1986a</td>
<td>68 newly presented, previously untreated patients. Normal basal GIP levels. Glucose tolerance (75g), significantly elevated plasma GIP levels seen at 30 - 90 minutes. Meal tolerance test (500kCal: 20% protein, 20% fat and 60% CHO) GIP response significantly higher in diabetic patients at several time points. This effect was independent of obesity.</td>
</tr>
<tr>
<td>Groop</td>
<td>1989</td>
<td>27 patients (9 lean, 18 obese). Treated by diet alone. Liquid test meal given (625kCal; 85g CHO, 27g protein and 21g fat). Basal GIP levels normal in diabetic patients. Lean diabetic subjects showed a reduced GIP response to the test meal. Obese diabetic subjects showed a similar GIP response to obese non-diabetic controls.</td>
</tr>
</tbody>
</table>
Table 1.2c Literature Review of Clinical Studies Investigating the Potential Involvement of GIP in Obesity

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Nature of study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creutzfeldt et al.</td>
<td>1978</td>
<td>125 obese subjects divided into 2 groups on the basis of their glucose tolerance. 3 tests were performed on 3 groups of subjects; test meal (152g CHO, 30g fat and 36g protein), oral glucose load (100g) and oral triglyceride (150ml corn oil suspension). Basal fasting GIP levels significantly elevated in obese patients. Obese patients gave a greater GIP response to the mixed meal and oral fat. In response to oral glucose the GIP levels were normal in obese patients.</td>
</tr>
<tr>
<td>Willms et al.</td>
<td>1978</td>
<td>147 subjects (&gt; 130% ideal body weight). Small groups of patients were subjected to an oral glucose load (100g) or a test meal (1031 Cal; 18g glucose, 14g maltose, 12g maltotriose, 52g lactose, 56g oligosaccharides, 100ml cream and 36g milk protein) or an oral triglyceride load (150ml corn oil suspension). Tests were administered before and after food restriction and in some cases starvation. Patients demonstrated increased basal levels and exaggerated GIP responses. These responses decreased significantly following food restriction and/or starvation.</td>
</tr>
<tr>
<td>Lauritsen et al.</td>
<td>1980</td>
<td>12 morbidly obese patients given a 50g oral glucose load. GIP release was normal.</td>
</tr>
<tr>
<td>Jones et al.</td>
<td>1989b</td>
<td>17 obese subjects. Subjects were given an oral glucose tolerance (75g) or a meal tolerance (500kCal; 20% protein, 20% fat and 60% CHO) and an iv glucose test (75g). All subjects received at least 2 of the 3 tests. Fasting GIP levels were normal. Post prandial GIP levels were significantly greater in the obese subjects. Following a period of dietary restriction and weight loss there was no change in GIP levels.</td>
</tr>
</tbody>
</table>

The awareness of the need to carefully control the study protocol has strengthened the evidence that a disordered entero-insular axis may be involved in the promotion of these three disease states. In insulin dependent diabetes a disordered entero-insular axis appears to be present in the short time between diagnosis and establishment of insulin therapy (Krarup et al., 1985). For non-insulin dependent diabetes and obesity, two disease states that often overlap, the abnormalities of the
entero-insular axis appear to be more fundamental. Weight loss in obese patients failed to normalise IR-GIP response to a test meal (Jones et al., 1989b). Abnormalities in IR-GIP secretion appear to be especially marked in obese individuals and obese non-insulin dependent diabetics. A strong association between GIP and insulin secretion was reported in obese American Indians as compared to the Caucasian population of America (Mazzaferri et al., 1985). Genetic components of obesity therefore influence the disorder of the entero-insular axis in both man and animals. GIP may therefore contribute to the apparent hyperinsulinaemia of obesity and non-insulin dependent diabetes under certain conditions.

If a disordered entero-insular axis is involved in these disease states, GIP is only one incretin of the endocrine arm of this regulatory control mechanism and so the specific role of GIP in disease states may be difficult to isolate from concomitant changes in other endocrine, paracrine or neural factors.

To date only two studies have been published investigating GLP-1(7-36)amide in non-insulin dependent diabetic patients (Hirota et al., 1990; Ørskov et al., 1991). Both studies found a significantly exaggerated IR-GLP-1(7-36)amide response to a 75g glucose load without the simultaneous suppression of the pancreatic C-terminal polypeptide which contains the GLP-1 sequence. Further work is required to confirm these results.

1.6.2 Animal Studies

The difference in meal size and composition and the influence of preceding diet on GIP response are factors which can be controlled far easier in animal studies. The entero-insular axis has been clearly demonstrated in rats and shown to respond to nutrient stimulation in a similar manner to the response seen in man (Kikuoka et al., 1987). Rodents can be genetically and chemically manipulated to demonstrate
obesity/diabetes syndromes which are comparable with human disease states. Several of these syndromes and their influence on the EIA are reviewed by Bailey & Flatt (1988 and 1991) and Bone (1991).

Animals with hypoinsulinaemic diabetes fall into two categories; those which develop diabetes spontaneously and those for whom the diabetes is chemically induced. The BioBreeding (BB) rat develops diabetes spontaneously as a result of massive B cell destruction by an autoimmune process. Evidence from studies indicates that specific dietary constituents may be important for the expression of diabetes in susceptible BB rats (Danemann et al., 1986; Scott, 1988). Investigations into the EIA and more specifically the GIP levels in these rats has not been undertaken. In contrast, induction of hypoinsulinaemia diabetes by administration of a large dose of streptozotocin has been shown to be associated with elevated plasma GIP in the fed state. An exaggerated GIP response to acute nutrient stimulation has also been reported in animals treated with streptozotocin (Bailey et al., 1986b).

Obesity-diabetes syndromes in animals have attracted more attention in this area of research. One extensively studied animal model is the genetically obese hyperglycaemic (ob/ob) mouse. The origins and characteristics of this animal are reported by Bailey et al. (1982). The model compares well with aspects of NIDDM in man. This is a spontaneously occurring syndrome with expression of the syndrome being influenced by environmental factors. Animals homozygous for the ob gene exhibit hyperphagia and severe obesity. Metabolically these animals are severely hyperinsulinaemic, hyperglycaemic and demonstrate insulin resistance (Bailey & Flatt, 1986). There is also strong evidence for a disordered entero-insular axis involvement in this syndrome as these animals display intestinal K cell hyperplasia and correspondingly elevated plasma GIP levels (Flatt et al., 1983; Flatt et al., 1984). The disorder of the entero-insular axis is likely to be an important contributor to the hyperinsulinaemia of this syndrome (Bailey & Flatt, 1988). Plasma IR-GIP levels are
particularly sensitive to dietary factors in these animals (Flatt & Bailey, 1984; Kwasowski et al., 1985), and age related changes in plasma GIP parallel changes in plasma insulin (Flatt et al., 1984). GIP levels are not responding purely to the hyperphagia in these animals as restricted feeding does not entirely normalise plasma GIP levels (Flatt et al., 1983).

The diabetes-obese (db/db) mice, another spontaneous syndrome, also exhibit entero-endocrine cell hyperplasia, with increased intestinal GIP concentrations (Flatt et al., 1983; Bailey et al., 1986a). In contrast, the fatty Zucker (fa/fa) rat, another spontaneous syndrome, has no change in GIP levels and only a moderate increase in insulin concentration (Morgan, 1979; Chan et al., 1984). However these animals are more sensitive to the actions of GIP. These animals show an increased GIP stimulated fatty acid incorporation into white adipose tissue (Beck & Max, 1986) and an increased sensitivity to the insulinotropic action of GIP (Chan et al., 1984).

Clinical and animal work, while not being conclusive, does suggest the possibility of a relationship between insulin and GIP levels and therefore a role for the EIA in the development or promotion of certain disease states. Investigations into a role for the EIA in disease has largely concentrated on the incretin effect. Both GIP and GLP-1(7-36)amide however, are directly biologically active in white adipose tissue metabolism. A contribution to obesity-diabetes syndromes via this pathway is therefore a strong possibility which requires further attention.

1.7 Rationale of Current Investigations

The aims of this thesis are to increase the breadth and depth of knowledge of the roles of two potent incretin hormones, GIP and GLP-1(7-36)amide, in both physiological and pathological states. All the states investigated were strongly associated with hyperphagia. The biochemical nature of this hyperphagia was not
questioned, animals were deemed to be hyperphagic when a sustained, significant increase in food consumption was observed. All investigations were performed in rodents in order to control the habitual diet and thereby avoid any difficulties in interpretation of the results due to dietary influences. The pathological conditions investigated if not spontaneous were induced by accepted, well documented methods.

Finally because GLP-1(7-36)amide has only relatively recently been identified, little is known of its importance in rats. Most of the evidence accumulated so far has been obtained from human and porcine studies. Little is known about the stimulus-secretion coupling of the L cell. Evidence that glucose stimulates its secretion has been obtained in humans and pigs by acute challenge experiments. To date only one study in dogs has reported stimulation of GLP-1(7-36)amide release by perfusion studies. An in-vitro stimulation procedure was utilised in rats to elucidate the nutrient requirements for GLP-1(7-36)amide release.
Chapter 2.0

METHODS
The following provides an account of the general procedures employed in this thesis. Actual experimental protocol is included, where necessary, in the relevant experimental Chapters.

2.1 Animals and their Housing Conditions

All albino, Wistar rats used were obtained from the Rodent Breeding Unit, University of Surrey. Transfer of animals, from the breeding unit to the experimental unit, was performed at least 24 hours prior to caging the animals for the individual experiments. NEDH rats for the insulinoma study were obtained from the experimental unit directly. Obese hyperglycaemic (ob/ob) mice and lean homozygous (+/+ ) litter mates were kindly donated by Dr C.J. Bailey, Dept. of Pharmaceutical Sciences, Aston University, Birmingham.

Animals were generally caged under similar experimental conditions (4-6 per cage), with sawdust bedding. Pregnant and lactating animals were caged individually with a sawdust and straw bedding. A standard 12hr photoperiod (0700-1900hr) at 22±2°C and 50% humidity was maintained. Food in the form of LAD1 (Biosure, Cambridge) and tap water were provided ad libitum. The composition of the diet was: 48% carbohydrate; 21.5% protein; 3.5% fat; 2.7% fibre; metabolisable energy 14.2MJ/kg. Where necessary food was withdrawn 18 hours before a provocative test was performed.

2.2 Administration of the Test Substances

Oral tolerances were assessed by straight tipped gavage administration of test substances intragastrically. Animals were removed from the cage immediately prior to dosing and held securely throughout the procedure which was performed as quickly as possible to cause minimum stress to the animal. The oral glucose load consisted of
3.2g D-glucose/kg body weight (51.2kJ/kg body weight) (BDH Chemicals, Poole). The glucose was solubilised (40% w/v) in RO (reverse osmosis purified) water.

The oral fat load consisted of 1.38g corn oil/kg body weight (51.2kJ/kg body weight) comprising on average 30% 18:1 and 50% 18:2 fatty acids (Paul & Southgate, 1976). The fat was administered in an emulsion with RO water. The emulsion was produced by 30 seconds sonication with an ultrasonic generator (Dawe Instruments Ltd.).

Intraperitoneal glucose tolerance tests were also performed in the insulinoma and streptozotocin studies. In all studies the concentration and total volume of the solution was the same as for the intragastric tests (8ml/kg body weight).

An insulin sensitivity test was performed, following an 18 hour fast, in the cold acclimation study and streptozotocin study. In all studies, porcine insulin (Actrapid-MC, Novo Industria, Copenhagen) was used at a dose of (1U/2ml) per kg body weight.

2.3 Standard Procedures

In all studies, food intake and body weight measurements were made on a daily or an alternate day basis as appropriate. Food consumption was taken as the difference between the amount of food provided and that left in the hopper 24 hours later. All animals were examined periodically for any signs of ill health.
2.4 Collection of Samples

2.4.1 Plasma

Blood samples were taken regularly, from control and test animals, from the cut tail tip. Animals were restrained during the procedure and every thought was given to avoid undue stress to the animal.

All blood samples were collected on ice in heparinised (sodium heparin 500IU/ml, BDH Chemicals Ltd, Poole) 250μl polyethylene microcentrifuge tubes (Elkay Products Inc., Shrewsbury). Tubes were heparinised by filling them with heparin solution and leaving it in contact with the tubes. The heparin solution was removed by pipette immediately prior to taking the blood sample. Samples were collected and spun immediately using a Beckman microcentrifuge for 30 seconds at 5000g. Plasma was immediately removed and aliquoted for glucose assay (10μl), insulin assay (20μl), and GIP assay (100μl). Samples were then frozen and maintained at -20°C until assayed. Thawing and refreezing of samples was avoided.

2.4.2 Tissues

Animals were killed by cervical dislocation and the small intestines (from the base of the fundus to the ileocaecal junction) from both control and test animals were excised, cleaned of extraneous material and weighed. They were then immediately placed on dry ice and subsequently transferred to a -20°C freezer prior to extraction into acid ethanol. Pancreatic tissues from ob/ob mice were also dissected and treated in a similar manner.
2.4.2.1 Extraction Procedure used for Tissue Extracts

Tissues were extracted according to the method developed by Kenny (1955). Acidified ethanol solution (750ml absolute ethanol; 250ml RO water; 15ml 36% v/v HCl) was added to the frozen tissue at a dose of 5ml/g wet weight in a universal glass tube. Pancreatic tissue from ob/ob mice was extracted at 10ml/g wet weight. Tissues were then cut to yield small pieces prior to sonication for 15 seconds with an ultrasonic generator (Dawe Instruments Ltd.). All procedures were performed in a cold tray at 4°C or by packing the sample tubes in ice. The resulting homogenate was subsequently incubated at 4°C for 24hrs prior to centrifuging (Beckmann J6) at 2500 rpm for 10 minutes. The supernatant was decanted and aliquoted into disposable tubes and frozen to -20°C for storage.

2.5 Plasma GIP RIA

This assay was developed by Morgan et al. (1978) and has subsequently been refined. Alterations were made where necessary, in the volume of plasma used and antiserum dilution, but the method was essentially unchanged.

2.5.1 Iodination of GIP

The method of iodination used was described by Kwasowski (1986) using the chloramine T (N-chloro p-methyl benzenesulphonamide) method of Greenwood & Hunter (1963).

Pure porcine GIP (originally a gift from Professor J.C. Brown, Vancouver, Canada and kindly provided by Dr. L. Morgan, University of Surrey) was used for the production of 125I-GIP. A stock supply of porcine GIP dissolved in phosphate buffer (pH 7.5, 0.4M) at a concentration of 5µg GIP per 10µl buffer was used. Aliquots
(10µl) of this solution were stored in plastic auto-analyser cups (Sterilin) at -20°C until required. The iodination procedure was carried out in a small volume and so great care was taken to ensure the small volume of sample was not disturbed from the bottom of the cup. The auto-analyser cup containing 10µl of GIP solution was allowed to equilibrate to room temperature before uncapping. The cup was then clamped and the lid carefully removed taking care not to disperse the small amount of solution around the sides of the cup.

A solution of chloramine-T (BDH Chemicals Ltd., Poole) in phosphate buffer (15µg in 10µl, pH 7.4, 0.4M) and a solution of sodium metabisulphite (BDH Chemicals Ltd., Poole) in phosphate buffer (40µg in 20µl, pH 7.4, 0.4M) were prepared immediately prior to iodination.

To the 10µl of GIP solution was added 1mCi (10µl) of 125I-sodium iodide (IMS30, Amersham International, Buckingham). Chloramine-T (10µl of the 15µg/10µl solution) was then added to the reaction vessel and the solution was carefully mixed for 15 seconds by alternate drawing up and expulsion of the reaction mixture into the pipette tip previously used to introduce the chloramine-T. The reaction was halted by the addition of 20µl of the sodium metabisulphite solution. The reaction products were immediately diluted with 200µl of a protein buffer: 0.1M sodium acetate pH 5.0, containing 0.5% (w/v) human serum albumin, HSA Fraction V, Sigma Chemical Co., Poole) and 500 KIU/ml aprotinin (Novo, Denmark).

The separation of reaction products was achieved by simple gel permeation chromatography. The reaction products were transferred to a 1.1x15 cm disposable polyethylene column (Wright/Amican provided by Dr. P. Kwasowski, University of Surrey) containing a matrix of superfine Sephadex G15 (Pharmacia LKB Biotechnology, Upsala, Sweden) which had been swollen at 4°C overnight in the protein buffer. The column was poured and equilibrated with the protein buffer prior to
the start of the iodination procedure. The column was eluted with the protein buffer. Fractions of approx 0.45ml (10 drops/fraction tube) were collected on a LKB Ultrorac 7000 fraction collector in disposable tubes. Approximately 24 fractions were collected. A pool of protein buffer 1-2cm in height was continuously maintained at the top of the column during the collection. 10µl of each fraction were transferred, by pipette, into disposable tubes and these aliquots and their pipette were counted for 10 seconds in a LKB 1260 Multigamma II.

Two peaks of radioactivity were obtained, the first containing the iodinated peptide, the second the free iodine. The highest fraction on the descending limb of the first peak was aliquoted (20µl aliquots) into LP3 tubes and stored at -20°C until required. The stability of the tracer was usually in the order of 6 to 8 weeks.

A typical elution profile of the iodination of porcine GIP is seen in Figure 2.1.0. It can be seen that approx 83% of total radioactivity measured is incorporated into the antigen peak (fractions 5-12). This varies within successful iodinations from 70-85% of the total radioactivity.

Figure 2.1.0 Typical Elution Profile of Iodinated Porcine GIP from a Sephadex G15 Column
2.5.2 Affinity Purification of Iodinated GIP

Iodinated porcine GIP was affinity purified on a column provided by Dr. P. Kwasowski. The column basically consisted of a partially purified IgG cut from a low avidity anti-porcine GIP antiserum covalently bound to an activated silica bed. Anti-porcine GIP IgG was partially purified by precipitation from serum with ammonium sulphate solution (50% w/v). The purified IgG was then covalently linked to approximately 1g of 100µm diameter porous activated silica beads, with a pore diameter of 1nm, using glutaraldehyde. The beads were then washed thoroughly in RO water and stored in a polyethylene column (1.1x15 cm) under carbonate/bicarbonate buffer pH 9.8 (sodium carbonate 1.59g/l and sodium hydrogen carbonate 2.93g/l with sodium azide 0.2g/l). By this method immunologically active $^{125}$I-GIP could be separated from immunologically damaged $^{125}$I-GIP. Affinity purification of $^{125}$I-GIP was carried out immediately prior to its use in the assay.

Affinity purification was carried out at room temperature in a radioactivity designated area. The column was clamped, allowed to settle, and cleaned with RO water (20ml) and then equilibrated with 20ml (in 4x5ml aliquots) of GIP assay buffer (0.04M phosphate buffer, pH 6.5, containing 0.5% w/v human serum albumin and 500 KIU/ml aprotinin). One or more of the 20µl aliquots of iodinated hormone were reconstituted to a total volume of 3mls with the assay buffer and layered on the column with a disposable pipette. The column was then topped up with assay buffer and capped. Continuous mixing of the column for 30 minutes at room temperature was performed using a rotormixer. After 30 minutes the column was reclamped, allowed to settle and then the buffer and damaged label allowed to run off. The silica bed was rewashed with 20ml (4x5ml) of RO water. Finally the bound labelled hormone was eluted by the addition of 5x1 ml of 0.5% v/v hydrochloric acid, pH 1.3. Affinity purified hormone was collected in a 5ml glass vial that had been previously washed with assay buffer. The vial once full, was transferred to a cold tray at 4°C. The
affinity column was further washed with 2×5ml RO water and then filled with carbonate/bicarbonate buffer (pH 9.8), stoppered and stored at 4°C.

Affinity purification waste (a 100µl aliquot), the empty fraction tube(s), and 100µl of affinity purified hormone were then counted in the 1260 Multigamma II for 30 seconds. A typical profile of proportional radioactivity is 30% affinity purified hormone, 40% waste and 30% still adsorbed to the original fraction tube. The problem of large amounts of radioactivity remaining in the fraction tube has very recently been addressed by the use of polyethylene tubes and dilution of fractions with 1ml assay buffer at the time of iodination prior to freezing.

Assessment of specific activity of the label was performed by self displacement. Standard curves were set up with increasing amounts of purified label present. The specific activity was in the order of 240-278 nCi/ng as estimated by calculating the concentration of IR-GIP in increasing amounts of labelled GIP added to the assay (Walker, 1977).

2.5.3 Preparation of Porcine GIP Standards

Pure porcine GIP was weighed on a microbalance (Perkin Elmer Autobalance) and dissolved in 0.1M formic acid containing 0.14M lactose, 0.04M HSA, 11mM citric acid, 6mM cysteine hydrochloride and 1600 KIU/ml aprotinin. 100µl aliquots (containing 1ng/µl porcine GIP) were freeze dried in glass vials to a constant vacuum. The vials were then stoppered without release of the vacuum and stored at -20°C until use. Aliquots were only defrosted once before use, and any remaining standard was discarded.
2.5.4 **Charcoal Stripped Serum**

Pooled fasting human serum was used to produce the charcoal-stripped serum (CSS). Charcoal (GSX, Sigma) was added to the pooled serum (20g charcoal/100mls serum). The mixture was stirred overnight (18 hours) at 4°C and then centrifuged (6000g for 30 minutes). The supernatant was then repeatedly filtered (Filtrox W-Steril Filters, H. Erben Ltd., Ipswich), under water suction, until all finings were removed and a pale coloured serum obtained. Aliquots (5ml) of CSS were stored at -20°C.

New batches of CSS and standards were always tested against the previous batch in the routine assay procedure and with quality controls to ensure reproducibility of results between assays.

2.5.5 **Antibody**

All assays were performed with rabbit anti-porcine GIP antiserum (RIC34/111J). The immunization procedure and characteristics of this assay are reported in Morgan et al. (1978). Comparison of this antibody with other published assays is reported by Jorde et al. (1983) and Krarup (1988). In brief, the rabbit was immunised with a GIP/ovalbumin glutaldehyde conjugate. The antiserum is very specific, showing less than 1% cross reactivity with: glucagon, VIP, secretin, pancreatic polypeptide, insulin or C-peptide. The antiserum recognises the 3 major molecular species of IR-GIP.

2.5.6 **Quality Controls**

Pooled postprandial and fasting plasma samples from rats covering the range of GIP concentrations measured in the standard curve were aliquoted and stored at -20°C. Each pool was assayed to determine the hormone concentration present (n=12 aliquots
per pool). Each quality control plasma was included (2 aliquots per pool) in the assays and the hormonal values obtained used to calculate within assay variation and between assay variation.

2.5.7 Assay Procedure

The GIP assay system employed was a 4-day equilibration assay. Antibody bound antigen was separated by immunoprecipitation using a donkey anti-rabbit gamma-globulin antibody.

The protocol for the assay is given in Table 2.1.
<table>
<thead>
<tr>
<th>Reagents added (µl)</th>
<th>Total Counts</th>
<th>Non-specific Binding (NSB)</th>
<th>Zero</th>
<th>Standards</th>
<th>Sample NSB</th>
<th>Sample</th>
<th>Quality control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
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<td>All tubes vortex mixed and incubated 24 hrs 4°C</td>
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<td>100</td>
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<tr>
<td>All tubes vortex mixed and incubated 4 hrs 4°C prior to separation of the bound and free phases</td>
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</tbody>
</table>

The diluent assay buffer used throughout the assay was 0.4M phosphate buffer pH 6.5, stored as a stock solution with 0.02% sodium azide. This stock was diluted 1:10 prior to use with RO water (giving 0.04M phosphate buffer) and 0.5% HSA (w/v) and 500 KIU/ml aprotinin (Novo, Denmark) were added.

Assays were set up in a cold tray at 4°C, all solutions being refrigerated before use. All standards were run in triplicate with samples being run necessarily only as single tubes. Non specific binding (NSB) tubes for samples were prepared using
pooled sample plasma and run at regular intervals in the assay. Therefore, each animal did not have its own NSB data. The NSB of sample plasma and CSS was always 3-6% of total binding.

In the setting up of the assay repeated volumes of buffer, label and antisera were added using a repeating multivolume pipette (Multipette 4780, Ependorf, Hamburg). In this assay the antiserum was used routinely at an initial dilution of 1:6000. The standards were set up in triplicate, by serial dilution, to cover the range 25-800 pmol/l.

Affinity purified labelled GIP was always added at a dilution giving approximately 7000-7500 cpm/100µl. Separation of the bound phase was achieved using a donkey-anti-rabbit γ-globulin antiserum, 50µl/tube, at an initial titre of 1:32 (Guildhay Antisera, Guildford). Normal rabbit serum (NRS) (Sigma) at an original titre of 1:135 was added to increase the subsequent size of the bound precipitate, (50µl/tube). Following the addition of NRS and the second antibody 100µl of 14% polyethylene glycol (PEG, theoretical molecular weight 6000) was added to accelerate the reaction. The incubation period in the presence of the second antibody was 4 hours. Tubes were then centrifuged, (20min. 2500rpm (1200-1300g) Beckmann J6 centrifuge). The resulting supernatant was aspirated from each tube using a water pump. The bound $^{125}$I-GIP remaining in the tube was then counted in a LKB 1260 Multigamma II. The parameter group for the assay was loaded from the permanent memory. The samples were loaded on the 12 hole counting racks in the order specified by the coding of the parameter setting. The Multigamma II was linked to a computer and data analysed and plotted using a spline function (% label bound against log concentration) best line fitting program. Unknown sample values were then automatically read off the standard curve.

A typical standard curve for the assay is shown in Figure 2.1.1.
Serial dilutions of mouse and rat plasma demonstrated parallelism with the standard curve (Figure 2.1.2).
2.5.8 Assesment of the Plasma GIP Assay

The sensitivity of the plasma GIP assay was determined in three ways. When calculated as the concentration of GIP equivalent to twice the standard deviation of the binding at zero the result was 21pmol/l. The approximate estimate of sensitivity given by the amount of unlabelled antigen required to produce a 10% fall in the binding at zero was 37.1pmol/l.

Intra-assay coefficient of variation was 2.5% at 27pmol/l; 4.7% at 134pmol/l and 8.5% at 402pmol/l. Inter-assay coefficient of variation was 2.9% at 28.1pmol/l; 5.6% at 133pmol/l and 10.2% at 406 pmol/l.

In Chapter 5, the serially sampled ob/ob mice plasma GIP assay was run with only 20μl of plasma per assay tube. The initial incubation volume in these tubes was maintained by adding an extra 80μl of assay buffer to all sample tubes. Charcoal stripped serum was not added to the standard tubes as the plasma effects of 20μl in 400μl are insignificant. The shortfall in volume of the standard tubes was made up with an extra 100μl of assay buffer. Subsequently all ob/ob plasma GIP results were multiplied by a factor of 5, to correct for the difference in plasma volume used. The sensitivity of the standard curve was modified by the alterations in assay protocol. Twice the standard deviation of the binding at zero was 35pmol/l and a 10% drop from zero binding was 51pmol/l. Inter-assay coefficient of variation was 7.3% at 31pmol/l; 13.2% at 92pmol/l and 21% at 387 pmol/l (n=12). Intra-assay coefficient of variation was 5.9% at 29pmol/l; 10.9% at 90pmol/l and 20% at 370pmol/l (n=4).

2.5.9 GTP Assay for Tissue Extracts

The assay for the measurement of IR-GIP in acid ethanol tissue extracts was modified from the plasma GIP assay procedure. The assay was essentially maintained
but CSS was no longer required, this being replaced by 100µl of assay buffer. The protocol is shown in Table 2.2. The standard curve was run in triplicate (Figure 2.4). Samples were run in duplicate. Serial dilutions of tissue extracts showed parallelism with the standard curve (Figure 2.4) and extracts were subsequently assayed at 1:100-1:200 times original dilution with assay buffer.

**Table 2.2 Protocol for Tissue Extracts GIP Assay**

<table>
<thead>
<tr>
<th>Reagents added (µl)</th>
<th>Total Counts</th>
<th>Non-specific Binding (NSB)</th>
<th>Zero</th>
<th>Standards</th>
<th>Sample NSB</th>
<th>Sample</th>
<th>Quality control</th>
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<td><strong>Day 4</strong></td>
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</tr>
</tbody>
</table>

All tubes vortex mixed and incubated 24 hrs 4°C

All tubes vortex mixed and incubated 48 hrs 4°C

All tubes vortex mixed and incubated 4 hrs 4°C prior to separation of the bound and free phases.
The limit of detection (2×SD from zero) was 28.6 pmol/l. The sensitivity of the assay assessed as a 10% drop from zero binding was 56.7 pmol/l. Intra-assay coefficient of variation was 8.3% at 57 pmol/l; 3.4% at 297 pmol/l and 2.6% at 790 pmol/l. Interassay coefficient of variation was 9.2% at 60 pmol/l; 4.6% at 312 pmol/l and 3.4% at 786 pmol/l.

2.6 GLP-1(7-36)amide RIA

Development of a sensitive, precise RIA to GLP-1(7-36)amide is difficult because the circulating concentrations of this hormone are so low. Kreymann et al. (1987) reported fasting levels in the order of 30-50 pmol/l. To date all the assay procedures reported (Ørskov et al., 1987a; Uttenthal et al., 1985; George et al., 1985; Kreymann et al., 1987) utilise extraction methods to concentrate the peptide levels prior to assay. When serially sampling rat plasma, sample volumes of >100 µl are not possible for any one experiment. To date no research group has been capable of...
measuring circulating concentrations of GLP-1(7-36)amide in serial samples taken from rats and other small laboratory rodents.

2.6.1 Iodination of GLP-1(7-36)amide

Synthetic human GLP-1(7-36)amide was purchased from Peninsula Laboratories Europe Ltd., St. Helens. The iodination procedure was exactly the same as that used for porcine GIP. A typical elution profile of iodinated GLP-1(7-36)amide is seen in Figure 2.1.4. In comparison to GIP iodination, it can be seen that a lower percentage of the iodine was incorporated into the GLP-1 molecule, typically 50-60% for a good iodination. This is due to less tyrosine residues in the amino acid sequence of GLP-1(7-36)amide for iodination.

Figure 2.1.4 Typical Elution Profile of Iodinated Human GLP-1(7-36)amide from a Sephadex G15 Column
2.6.2 **Affinity Purification of Iodinated GLP-1(7-36)amide**

An affinity column for the purification of labelled GLP-1(7-36)amide was provided by Mr. R. Elliott, University of Surrey. This column was similar to the GIP column and the procedure for purification identical.

2.6.3 **Preparation of Human GLP-1(7-36)amide Standards**

Human GLP-1(7-36)amide standards were produced in exactly the same way as the GIP standards.

2.6.4 **Production of GLP-1(7-36)amide Antiserum**

GLP-1(7-36) amide, like porcine GIP, is on its own a poor immunogen. It therefore requires to be linked to a larger and therefore more immunogenic protein to promote a stronger immune reaction in the host animal. Numerous reagents are available to couple small proteins (haptens) to larger proteins. Two traditional methods were used to produce conjugates of GLP-1(7-36)amide and bovine serum albumin (BSA, Fraction V, Sigma, Poole).

2.6.4.1 **GLP-1(7-36)amide-BSA Conjugation with Carbodiimide**

This method was modified from that first reported by Goodfriend *et al.* (1964).

Conjugation procedure:

200 µg of synthetic, human GLP-1(7-36)amide (Bachem, Sweden) was dissolved in 200 µl of RO water. To the peptide solution, bovine serum albumin (fraction V 450 µg in 200 µl RO water) was added.
1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (560µg/1ml of RO water) was then added dropwise whilst stirring. This gave an initial peptide:protein:ethyl-CDI ratio of 1:0.067:42 as suggested by Rehfeld et al. (1972). The reaction mixture was gently stirred at 20°C for 18 hours (overnight). The resultant slightly opalescent solution was dialysed against 4×11 RO water at 20°C.

The GLP-1(7-36)amide-BSA conjugate solution was adjusted to 2ml volume with RO water and stored in 0.5ml aliquots at -20°C until required.

2.6.4.2 GLP-1(7-36)amide-BSA Conjugation with Glutaraldehyde

This method was derived from that reported by Reichlin et al. (1968).

Conjugation procedure:

Conjugation was carried out immediately prior to immunization of the animals. GLP-1(7-36)amide (100µg in 100µl RO water) was mixed with a BSA solution (170µg in 50µl RO water). To this was added 150µl of a 1:10 dilution of an original 25% aqueous solution of glutaraldehyde (Glutaraldehyde Grade 2, Sigma, Poole). The reactants were thoroughly mixed by pipette and allowed to rest at room temperature for 60 minutes.

2.6.4.3 Immunization Procedure

Rabbits were the preferred host animal for immunization. Four young, female, half lop rabbits were obtained (Ranch Rabbits Ltd, Crawley Down) and caged at the Breeding Unit, University of Surrey. On the day of priming, each rabbit was calmed with 0.1ml of Hipnorni. Each rabbit was bled by the ear vein prior to immunization and approximately 10ml of blood per rabbit was collected and allowed to clot. Serum
obtained from this retracting clot was stored at 4°C with sodium azide (0.1% w/v) and used in subsequent assays to assess non-specific binding of the antiserum.

Two rabbits were immunized with the carbodiimide linked conjugate and two with the gluteraldehyde linked conjugate. Each rabbit received 0.5ml of the conjugate (assuming total conjugation of the peptide, a dose equivalent to 50µg GLP-1(7-36)amide) which was initially mixed with 100µl of BCG (BCG Vaccine BP, BNF, Intradermal). Immunogen solutions were thoroughly emulsified with marcol (two volumes marcol:one volume aqueous immunogen) prior to injection. Immunization was carried out intradermally at 30 to 40 sites (approximately 50µl emulsion/site) in the subscapular region.

Monitoring of the immunological response and subsequent boosting of the animals was carried out by Mr. R. Elliott and Dr. L. Morgan. After several months successful antiserum dilution curves with reasonable titres were obtained from 3 of the 4 rabbits. One bleed from a rabbit, immunized 130 days previously, with a glutaraldehyde conjugate was kindly donated by Dr. L. Morgan and Mr. R. Elliott. This rabbit (coded G2) had produced the most marked immunological response to the primary immunization, but had subsequently died (approx 150-200 days post immunization) through undetermined causes before any immunization boosts had been administered.

For comparative purposes Professor J.J. Holst (Institute of Medical Physiology, Copenhagen, Denmark) kindly donated an antiserum raised against GLP-1(7-36)amide by his research group (Antiserum Code No 2135). The characteristics of this antiserum are reported in Ørskov et al. (1987a). Briefly this antiserum was raised in rabbits against a GLP-1(7-36)amide/BSA carbodiimide conjugate. It cross reacts 100% with synthetic GLP-1(1-36)amide but not with GLP-2
or glucagon. It also cross reacts, showing a high affinity for the large C-terminal proglucagon pancreatic polypeptide.

2.6.4.4 Comparison of Antisera and Assay Development

Antiserum 2135, was provided as a purified antiserum (50µg) freeze dried. The antiserum was reconstituted in 50µl phosphate buffer (0.04M, pH 6.5) at 4°C and then further diluted with phosphate buffer to give 1:100 dilution of the original solution. This was aliquoted (100µl aliquots) into disposable tubes, capped and stored frozen at -20°C. Thereafter thawing and refreezing of aliquots was avoided.

Antiserum G2 (15.5.89) was not purified from the original serum obtained at that bleed time. The serum was maintained at 4°C with 0.1% w/v sodium azide.

GLP-1(7-36)amide and GIP are very similar hormones in many respects, therefore, for convenience it was decided to investigate and develop a GLP-1 assay under the normal assay conditions used successfully for GIP.

The initial assessment of the antisera was made by investigating the titre of the antisera by means of antiserum dilution curves.

Production of Antiserum Dilution Curves

For each antiserum, duplicate tubes were set up containing 200µl assay buffer (0.04 M phosphate buffer, pH 6.5, containing 0.5% w/v human serum albumin and 500 KIU/ml aprotinin). In addition, 100µl of iodinated GLP-1(7-36)amide (affinity purified and diluted to approx 7000 cpm/100µl) was added to each tube. Antiserum 2135 (100µl) was added at an initial dilution of 100, 1000, 2000, 4000, 8000, 16000 and 32000 times in assay buffer. Antiserum G2 (15.5.89) was diluted initially 125.
250, 500, 1000, 2000, 4000 or 8000 times, again in assay buffer. Phase separation of tubes was achieved by adsorption (1ml/tube) using dextran coated charcoal. A 20% v/v solution of 5% w/v Dextran T-70 coated charcoal (Sigma) in phosphate buffer pH 6.5, 0.04M.

Dilution curves of pooled normal rabbit serum were also run over the range appropriate for each antiserum to check the non-specific binding of the tubes under these experimental conditions. The resultant mean antiserum dilution curves are given in Figure 2.1.5.

Figure 2.1.5 Mean Antiserum Dilution Curves for Antisera 2135 and G2

Values are means of n=4 observations.

![Antiserum Dilution Curves](image)

The maximum binding of affinity purified, iodinated GLP-1(7-36)amide with excess antibody was typically 70-80%. The titre of each antiserum was therefore taken to be the initial dilution that would bind 35% of the added label under the conditions of the assay. Antiserum 2135, therefore, had a titre of an initial dilution of 1:10,000, comparing favourably with the titre reported by Ørskov et al. (1987a) and G2 (15.5.89) had a titre of 1:2000.
Production of Standard Curves

The assay procedure is given in Table 2.3.

**Table 2.3  Protocol for Tissue Extract GLP-1(7-36)amide Assay**

<table>
<thead>
<tr>
<th>Reagents added (µl)</th>
<th>Total Counts</th>
<th>Non-specific Binding (NSB)</th>
<th>Zero</th>
<th>Standards</th>
<th>Sample NSB</th>
<th>Sample</th>
<th>Quality control</th>
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</thead>
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<td>All tubes vortex mixed and incubated 24 hrs 4°C</td>
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<tr>
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<td>Donkey anti-rabbit gamma globulin</td>
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The assay conditions were the same as for the tissue extracts GIP assay. Antisera were run at their half-maximum binding titre dilutions as mentioned previously. In addition to charcoal separation of phases the double antibody procedure was also tested at the same dilution used for the tissue GIP assay. In total four standard curves were run, two using antiserum 2135 and two using antiserum G2 (15.5.89). The results are given in Figure 2.1.6.

**Figures 2.1.6** Assessment of Two Different Methods for the Separation of Phases

**Antiserum 2135**

<table>
<thead>
<tr>
<th>% Label Bound</th>
<th>Human GLP-1(7-36)amide concentration (pmol/l)</th>
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<tbody>
<tr>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
</tr>
<tr>
<td>30</td>
<td>800</td>
</tr>
<tr>
<td>20</td>
<td>1200</td>
</tr>
<tr>
<td>10</td>
<td>1600</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- Charcoal Separation
- DAR Separation

**Antiserum G2**

<table>
<thead>
<tr>
<th>% Label Bound</th>
<th>Human GLP-1(7-36)amide concentration (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
</tr>
<tr>
<td>30</td>
<td>800</td>
</tr>
<tr>
<td>20</td>
<td>1200</td>
</tr>
<tr>
<td>10</td>
<td>1600</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- Charcoal Separation
- DAR Separation
From the standard curves it can be seen that higher binding of label to antiserum was recorded using the second antibody method of separation. This would therefore allow lowering of the initial antiserum dilution, to maintain the 35% titre, producing a more sensitive assay. The initial antiserum dilution was therefore adjusted to 1:12000 (for antiserum 2135) and 1:2500 (for G2) and immunoprecipitation became the method of choice for separation of the bound and free label. Typical assay standard curves are shown in Figure 2.1.7.

As a consequence of the heightened sensitivity of the assay obtained using an immunoprecipitation method of phase separation, reducing the second incubation time from 48 to 24 hours was investigated. However reducing the time course of the assay, while maintaining the reaction at 4°C, greatly reduced its sensitivity (data not shown) and therefore the idea was abandoned.
Figure 2.1.7  Typical Standard Curves for Human GLP-1(7-36)amide Immunoreactivity

Values are mean and standard deviation for 6 replicates

Antiserum 2135

Assessment of Specificity of the Antisera

Specificity was assessed by investigating the cross reactivity of the antisera with a range of gastrointestinal hormones, glucagon and the synthetic parent molecules, GLP-1(7-37) and GLP-1(1-36)amide (all hormones were obtained from Penninsula). These
hormones were incubated individually with the antiserum in the standard assay procedure over a range of concentrations; 150 pg/ml to 50ng/ml in the case of GLP-1(7-37); 2.5-1000ng/ml for glucagon, GLP-2, human GIP, VIP, secretin, somatostatin and motilin. The results are given in Table 2.4.

Table 2.4  Demonstration of Specificity of the Assay

<table>
<thead>
<tr>
<th>Hormones Tested</th>
<th>Antiserum 2135</th>
<th>Antiserum G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1(7-37)</td>
<td>87%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>GLP-1(1-36)amide</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>GLP-2</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>VIP</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Human GIP</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Secretin</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Motilin</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Glucagon</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

From the results it can be speculated that antiserum 2135, as Ørskov et al. (1987a) described, is a sideways looking antiserum binding towards the N terminal of the GLP-1(7-36)amide molecule. This antiserum is therefore unable to distinguish between the original and the truncated forms of the molecule. G2 (15.5.89) on the other hand must have a binding region towards the C-terminal region involving or consisting of the final amidated arginine residue of the molecule.

Serial dilutions of intestinal extracts from several experiments were run in order to show parallelism with the standard curve (Figure 2.1.8) and from these serial dilutions quality control tissue samples were obtained to subsequently monitor the assay.
Values are mean ± SD for n=6 observations

Parallelism between human IR-GLP-1(7-36)amide and IR-GLP-1(7-36)amide in tissue extracts from streptozotocin diabetic, insulinoma-bearing and neonatal rats is also demonstrated. Values are means of 6 samples.

Antiserum 2135

Antiserum G2

Having established the differing specificities of the two antisera, the characteristics of the assay were established with the running of several standard curves and quality control intestinal extracts. Antiserum G2 had a sensitivity of 18pmol/l
(2×SD from zero) or 30pmol/l (10% reduction in zero binding). The intra-assay coefficient of variation was 3.1% at 24pmol/l; 9.2% at 538pmol/l and 15.7% at 1365pmol/l. The interassay CV was 5.7% at 25pmol/l; 8.6% at 549pmol/l and 16.6% at 1343pmol/l. Antiserum 2135 had a sensitivity of 22pmol/l (2×SD from zero) or 34pmol/l (10% reduction in zero binding). The inter- and intra-assay CV were very similar to those obtained with antiserum G2.

In order to prove no additional interference from other hormones in the tissue extracts, gel filtration of extracts was performed.

2.6.5 Gel filtration of Intestinal Extracts

Prior to gel filtration, extracts (2ml aliquots) were necessarily dried down to remove the acid ethanol. Aprotinin (500 KIU/ml) was added as a precaution to the extracts prior to removal of acid ethanol. Extracts were blown dry under nitrogen at room temperature for 30 minutes to evaporate the ethanol and then transferred to polypropylene centrifuge tubes (Falcon) and placed in a rotary evaporator for 2 hours until all liquid was evaporated and a yellow precipitate remained. The precipitate was reconstituted in 2ml 0.5M acetic acid for subsequent gel filtration.

Gel filtration was performed by modification of the method of Holst et al. (1987). A single jacket glass column (1.5cm×100cm) was clamped above a fraction collector. The whole apparatus was prepared and maintained at 4°C. The column was packed with Sephadex G50, fine grade (Pharmacia LKB Biotechnology, Upsala, Sweden). The Sephadex was previously swollen for 24 hours at 4°C in an excess of 0.5M acetic acid (40g/l). The column was packed manually in a single action with the Sephadex slurry. It was then allowed to settle for 4 hours and equilibrated with 2 litres of 0.5M acetic acid. Flow rate was adjusted to 30ml/hour. The Sephadex bed settled to a height of 68cm providing a total bed volume of 481cm³. A reservoir of eluent was
maintained at the head of the column bed by means of a peristaltic pump. Fractions of 2ml volume were collected by means of a fraction collector (LKB Ultrarack 7000).

The column was calibrated by means of gel filtration standards (Sigma). In addition, cyanocobalamin (Vit B₁₂, Sigma) was run as a low molecular weight marker. Standards were run at the concentrations advised, varying from 2mg to 5mg/ml in 0.5M acetic acid. 10% glycerol was added to all standard solutions to aid loading of sample. Standards were loaded over 72 hours in 3 separate runs; dextran blue was run in isolation followed by albumin, cytochrome C and cyanocobalamin, and finally carbonic anhydrase and aprotinin. The maximum sample volume loaded at any one time was 2ml. Samples were loaded onto the bed surface under the reservoir of eluent by means of a fine (1mm diameter) polyvinylchloride tube attached to the loaded sample syringe. The calibration curve obtained is shown in Figure 2.1.9. Kav is a partition coefficient relating the volume eluted from the start of sample application to the elution peak (Ve) minus the elution volume for the excluded component (Vo) to the total volume (external and internal) available (Vt). \( Kav = \frac{(Ve-Vo)}{(Vt-Vo)} \).
The total volume of the packed bed was established using a 4mg/ml solution of copper (II) sulphate. All coloured compounds were seen to progress through the column as strong horizontal bands. Eluted fractions were monitored spectrophotometrically, in quartz cuvettes, at 280nm using a Uvicon Spectrophotometer.

Fractions collected from the subsequent gel filtration of intestinal extracts were frozen to -80°C and freeze dried (Edwards Freeze Dryer) until a constant vacuum was maintained. Subsequently some fraction tubes were seen to contain white crystalline powder others were seen to be empty. All tubes were reconstituted to 2mls volume with assay diluent on the day of assay and assayed, as per GLP-1 assay procedure, at an initial dilution of 1:10 and 1:40. All extracts were assayed with both GLP-1(7-36)amide antisera.

Figure 2.2.0, demonstrates the typical gel filtration profile obtained with 4 intestinal extracts from experimental rats. In addition intestinal and pancreatic extracts of ob/ob mice were also filtered. These results will be given in Section 5.3.4.1.
In conclusion, the two antisera appear to detect similar levels (within 15% error) of IR-GLP-1(7-36)amide in intestinal extracts. Therefore in subsequent studies, due to the limited supply of Antiserum 2135 only Antiserum G2 was used to measure.
intestinal IR-GLP-1(7-36)amide levels. The results indicate that GLP-1(7-37) is present in intestines in negligible amounts and that rapid conversion to the amidated form must be the normal procedure.

2.7 Plasma Insulin RIA

Insulin was measured, again by RIA, using a method modified from Albano et al. (1972), as described by Flatt & Bailey (1981).

2.7.1 Insulin Standard

A crystalline mouse/rat insulin standard, (Novo Industria, Copenhagen), was reconstituted to 1mg/ml in 0.4M phosphate buffer, (pH 7.4). 150µl aliquots were frozen to -20°C and maintained at this temperature until use. On the day of assay the standard was defrosted and diluted to give a top standard of 20ng/ml, in 0.04M phosphate buffer (1:10 dilution of stock 0.4M phosphate buffer) pH7.4, containing 0.5%w/v bovine serum albumin and 500KIU/ml aprotinin. This top standard was serially diluted with buffer to give a final range of standards from 0.039-20ng/ml.

2.7.2 Iodinated Insulin

Iodinated bovine insulin (3µCi/5ml) manufacturers specific activity of 50µCi/µg, was supplied by Amersham International, Buckingham. This was stored at 4°C and diluted 1:20 with assay buffer at the time of assay giving a concentration of 125I-insulin of 1ng/ml.
2.7.3 Quality Control Plasma

Quality control pooled plasmas were obtained from fasted and fed (3.2g glucose/kg body weight) rats, aliquoted and frozen to -20°C. Duplicates of each pool of plasma were run in each assay to check within and between assay variation.

2.7.4 Antiserum

The antiserum used (Flatt & Bailey, 1981) was raised in guinea-pig against crystalline porcine insulin, (Antiserum P2, 4/11/76). Antiserum was stored at -20°C, in 100µl aliquots of a 1:100 dilution in phosphate/albumin buffer. At the time of assay one aliquot was diluted to 45ml with assay buffer giving an initial dilution of 1:45,000. This titre of antiserum gave a maximum binding of labelled insulin of 35%.

2.7.5 Assay Procedure

The protocol for the insulin assay is given in Table 2.5.
Table 2.5  Protocol for Plasma Insulin Assay

<table>
<thead>
<tr>
<th>Tubes routinely set up</th>
<th>Reagents added (µl)</th>
<th>Total Counts</th>
<th>Non-specific Binding (NSB)</th>
<th>Zero</th>
<th>Standards</th>
<th>Sample</th>
<th>Quality control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay diluent</td>
<td></td>
<td>300</td>
<td></td>
<td>200</td>
<td>100</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Antiserum</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>QC plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>All tubes vortex mixed and incubated 24 hrs 4°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td></td>
<td>100</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$^{125}\text{I}$- Insulin</td>
<td></td>
<td>All tubes vortex mixed and incubated 48 hrs 4°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td></td>
<td>1000</td>
<td></td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Dextran coated charcoal</td>
<td></td>
<td>All tubes incubated 20 minutes 4°C prior to separation of the bound and free phases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All procedures were performed at 4°C in a cold tray and all solutions refrigerated prior to use. Standard tubes were run in triplicate. Samples were necessarily run individually. The assay is an equilibrium reaction over 4 days, the first 24 hours being the incubation of the antiserum with the unlabelled antigen, without the presence of labelled antigen.

Separation of phases was achieved by adsorption using dextran, (T-70), coated charcoal, (a 1:5 dilution of 0.5% dextran T-70 stock 5% charcoal [Sigma] solution). The solution being diluted with phosphate buffer pH 7.4, 0.04M.
Charcoal stripped serum was not run in the assay as 20µl of plasma in an initial incubation volume of 300µl does not contribute significantly to non-specific effects and negates the one potential problem with the assay method in that charcoal separation is relatively sensitive to non-specific effects.

The sensitivity of the assay as assessed by 2×SD from zero binding was 0.087ng/ml and assessed by a 10% drop in zero binding was 0.105ng/ml. Quality control plasmas indicated that the intra-assay coefficient of variation was 4% at 0.197ng/ml; 8.4% at 1.320ng/ml; 7.7% at 5.610ng/ml and 3.1% at 10.75ng/ml. The inter-assay coefficient of variation was 5.1% at 0.203ng/ml; 8.4% at 1.41ng/ml; 9.2% at 5.72ng/ml and 5.1% at 10.49ng/ml.

2.8 Plasma Glucose Assay

Glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyser II (Stevens, 1971).

The analyser utilises the principle that when glucose is oxidised by glucose oxidase the rate of oxygen utilisation is proportional to the glucose concentration.

\[
\text{glucose oxidase} \\
\beta\text{-D-glucose} + O_2 \rightarrow \text{Glucuronic acid} + H_2O_2
\]

To prevent the \( H_2O_2 \) from yielding back any oxygen to the solution two peroxide-dependent reactants are present.

The rate of \( O_2 \) utilisation is monitored by an oxygen electrode, located within the side of the reaction vessel, which responds to the concentration of \( O_2 \) in the reaction.
solution. The electrode compares the rate of use of an unknown sample, with that of a standard glucose solution of 8.3 mmol/l.

Procedure

The machine is calibrated to provide a repeatable digital oxygen display reading of 23.5 with a freshly aerated supply of Beckmans Glucose Reagent at 37°C. Aeration is achieved by shaking the bottle of reagent 2-3 times with 2-3 charges of air. Calibration with the standard glucose solution (8.3mmol/l) (Beckman Glucose Standard) is then performed by introduction of 10µl standard solution using a micrometer pipette (Gilson) into the reaction cup of the analyser. Glucose reagent is already present in the reaction cup and so the reaction proceeds immediately. The reaction solution is mixed by alternate drawing up and expulsion of the solution from the pipette used for glucose standard introduction. The pipette is subsequently removed and a reading noted from the digital display. The analyser automatically goes through a wash cycle to ensure fresh reagent is available for the next aliquot of standard.

Once calibrated, the glucose concentration of 10µl samples of defrosted plasma can be measured. The analyser will theoretically recover within ±3% of the nominal value of glucose standards up to 25mmol/l. For samples which exceeded this value the procedure was repeated using 5µl samples rather than diluting the sample prior to analysis. This was especially important for the streptozotocin diabetic rats and the ob/ob mice which suffer severe hyperglycaemia. Wherever possible all samples from a single study were analysed in one run. The precision of glucose standards within runs had a standard deviation (SD) of 0.1 mM for 20 replicates and from day to day analysis the SD was 0.2 mM.
Data Handling and Statistical Analysis

Normal distribution of the data were confirmed using Kolmogorov-Smirnov's one sample goodness of fit test. Variances, where necessary, were stabilised by transforming the data to $\log_{10}$. Data obtained from sequential sampling of blood over a specific time period were assessed by means of repeated measures analysis of variance. Variables of feeding regime and in the case of the cold acclimated animals the environmental temperature were considered in the analysis.

Descriptive data for three or more treatment groups was obtained using one-way analysis of variance followed by a range test. Where group sizes were equal Duncan's multiple range test at the 0.05 and 0.01 significance level was used. Where group sizes were unequal Scheffe single range test was used, at the 0.05 and 0.01 significance level, Scheffe being exact for unequal group size.

Descriptive data for two treatment groups were obtained using two-tailed Student's unpaired or paired t-test at the 0.05 and 0.01 significance level as appropriate. Differences over time in an individual group were assessed by Student's unpaired t-test at the same significance levels.

Incremental responses were calculated by subtracting response at time 0 from response at time 30. Integrated responses were calculated by measuring responses $(r_1...r_n)$ at discrete time intervals $(t_1...t_n)$ and calculating the area under the graph minus the basal area $(r_1t_0)$. Integrated response was calculated as the sum of the integrated responses between all adjacent discrete measurements, and the final sum divided by two to give the final expression as $(\text{mmol/l}) \cdot \text{hr}^{-1}$, $(\text{ng/ml}) \cdot \text{hr}^{-1}$, or $(\text{pmol/l}) \cdot \text{hr}^{-1}$. One-way analysis of variance followed by Duncan's multiple range test were employed to assess significance between three groups. Student's unpaired t-test was employed to assess significance between two groups.
Chapter 3.0

BASAL AND NUTRIENT STIMULATED PLASMA IR-GIP AND INTESTINAL ENTERO-ENDOCRINE FUNCTION IN PHYSIOLOGICAL STATES ASSOCIATED WITH HYPERPHAGIA
The following studies all concern physiological states associated with short-term hyperphagia. The degree of the hyperphagia varies as does the underlying cause of the increased food intake. The aims in all five studies were to investigate whether normal K cell secretory function was modified by the increased physiological stresses of changing the environmental conditions and/or food availability or reproductive status. Each study will be considered separately and overall conclusions will be discussed at the end of this Chapter.

3.1 The Effects of Pregnancy and Lactation upon Basal and Nutrient Stimulated Plasma IR-GIP and Intestinal IR-GIP and IR-GLP-1(7-36)amide

3.1.1 Introduction

Pregnancy in humans is a physiological state where food intake, both in terms of the quantity of food consumed and its nutrient composition, is acknowledged to be modified (Durnin, 1987). Glucose homeostasis is also known to be modified, fasting plasma glucose concentrations being reportedly decreased (Lind et al., 1973), or maintained (Kühl, 1975) in the presence of a substantially increased plasma insulin concentration (Kühl, 1975). An increased plasma insulin response to an oral glucose load has been described in late pregnancy (Kühl, 1975; Hornnes et al., 1978). In contrast, the incretin effect has been reported to be reduced in late pregnancy (Hornnes et al., 1979). Two groups of researchers have investigated whether this is due to modification of GIP secretion. Plasma IR-GIP levels measured during late pregnancy and early post partum have shown a reduced IR-GIP response to nutrients during the later stages of pregnancy (Hornnes et al., 1979; Jenssen et al., 1988; Hornnes et al., 1978). The two research groups differ in their findings as to whether basal fasting GIP levels are modified by pregnancy. This is perhaps due to their experimental design which utilised different times post partum to obtain control values. Use of non-
pregnant age matched control subjects suggests a reduction in plasma IR-GIP concentration at 36 weeks of gestation (Berseth et al., 1990).

Animal experiments dealing with pregnancy and lactation are mainly concerned with protein energy malnutrition and its effect on the viability of the offspring. Hyperphagia during pregnancy and lactation in rats has been noted (Cole & Hart, 1938; Campbell & Fell, 1964; Larralde et al., 1966; Craft, 1970). Gastrointestinal physiology has been shown to be modified in pregnancy and lactation as a result of the hyperphagia (Boyne et al., 1966; Craft, 1970). To date no studies have been reported on the incretin effect in animals during pregnancy or lactation. However, we have previously shown modified intestinal IR-GIP concentrations in the small intestines of mice under conditions of altered reproductive status (Knapper et al., 1989, unpublished work).

The aims of this study were to look at how pregnancy and lactation in the rat could potentially alter the normal functioning of the GIP secretory cells in both the basal state and in response to acute nutrient stimulation. Small intestinal concentrations of IR-GIP and IR-GLP-1(7-36)amide were also measured in late pregnancy and at the height of lactation for comparative purposes.

3.1.2 Methods

3.1.2.1 Animals and their Treatment

Thirty virgin, female, albino Wistar rats were used in this study. The origin of these animals was discussed in Section 2.1. The test animals (n=18) were mated overnight and then caged individually once pregnancy was confirmed, by external examination on day 8. Pregnancy was allowed to proceed without intervention and on day 22 post coitus the animals were allowed to litter naturally. Litter sizes were
standardised within 24 hours of birth to n=10, normal litter size for this colony being 8-
10 animals. The housing conditions and dietary provision for the animals were
discussed in Section 2.1.

3.1.2.2 Experimental Procedure

Food intake and body weight measurements were monitored on alternate days
as described previously (Section 2.3). Plasma, for the analysis of basal glucose, IR-
insulin and IR-GIP levels, was obtained at regular intervals during pregnancy and
lactation from 6 test and 6 control animals and treated as stated previously (Section
2.4.1). On day 21 of pregnancy and day 16 of lactation acute nutrient response tests
were performed. These consisted of an oral glucose tolerance challenge (51.2 kJ/kg
body weight) or an oral fat challenge (51.2 kJ/kg body weight). The exact nature of
these tests has been described previously (Section 2.2). Pilot studies, using non-fasted
animals (so as not to cause undue stress of the animals at such a late stage of their
pregnancy) revealed problems with dosing fed hyperphagic animals and large
individual variations in response to the test. No animal littered early and all produced
successful litters (7-21 offspring) on day 22 of gestation. Due to the problems of
dosing the animals it was decided to fast the animals for 18 hours prior to the acute
nutrient tests. Following fasting and an acute nutrient challenge only 7 of a total
number of 12 pregnant rats produced successful litters (ranging in size from 10-17
animals). The remaining 5 animals cannibalised their offspring immediately after
parturition. These animals were immediately withdrawn from the study and replaced
by 5 new rats of a similar age with litters of a similar age for use during the subsequent
tests. In all subsequent acute nutrient tests the responses from the original rats (n=7)
and the replacement rats (n=5) were statistically indistinguishable.
In addition to the basal and nutrient stimulated measurements, on day 20 of pregnancy and on day 21 of lactation, small intestines were excised from a group of control (n=6) and test (n=6) rats as previously described (Section 2.4.2).

3.1.3 Results

3.1.3.1 Body Weight and Food Intake

Mean body weight results have been calculated for every 2-3 days of the study. The results are shown in Figure 3.1.1.

Repeated measures analysis of variance revealed that body weight increased significantly in both the control and test animals over the study period (p<0.01). The rate of change in body weight was however greater in the test animals (repeated measures between group difference, p<0.01). Analysis of individual time points revealed that the pregnant animals became significantly heavier than age matched controls at 13 days post coitus (p<0.01). The large fall in body weight at the end of pregnancy was due to the effects of fasting the animals prior to the acute nutrient tolerance tests and parturition. A smaller, transient fall in body weight was also seen in the control animals at this time due again to the effects of the experimental procedure.

Immediately following parturition body weights of control and test animals were similar. Body weight increased in the test animals as lactation became established and the demands of the offspring increased. Differences in body weight between the two groups became significant at several time points. From day 16 post partum onwards mean body weight gain in the lactating animals levelled off and started to decline slowly. By the end of lactation there were no significant differences in body weights between the two groups.
Figure 3.1.1 The Effects of Pregnancy and Lactation on the Body Weight of Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Parturition occurred day 22 post coitus. Symbols (*) indicate values significantly different from control values at that time point when assessed by Student's unpaired t-test. *=p<0.05;

Mean food intake is summarised graphically in Figure 3.1.2.
The Effects of Pregnancy and Lactation on the Food Consumption of Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Parturition occurred day 22. Symbols (*, **) indicate values significantly different from control values when assessed by Student's unpaired t-test, *=p<0.05; **=p<0.01. From day 24 onwards the differences between the two groups were always statistically significant, p<0.01 (symbols not shown).

It can clearly be seen that although pregnant animals are hyperphagic (maximum food consumption 1.5 × control animals intake) lactating animals consume significantly greater quantities of food, (maximum food consumption 3.6 × control animals intake). The repeated measures analysis of variance within group difference was p<0.01. The fall in food intake day 20-22 in the pregnant animals is due to fasting for 18 hours and parturition. During lactation, maximum food intake was seen on days 33-37 post coitus (days 12-16 post partum). Food intake then started to fall as weaning approached but the animals still demonstrated a clear degree of hyperphagia at this stage.
3.1.3.2 Basal Levels of Plasma Glucose, IR-Insulin and IR-GIP During Pregnancy and Lactation in Wistar Rats

The mean results for plasma glucose, IR-insulin and IR-GIP are shown in Figure 3.1.3. Blood was sampled on the same days post coitus and post partum. Samples were run in a single assay. Control values during pregnancy and lactation were similar (means within 1 SD of each other) and so the control data were pooled to give one result.

Fed plasma samples were obtained at 3 time points during gestation (post coitus) and lactation (post partum) on days 8, 15 and 20, to obtain basal measurements and to maintain familiarity of the animal with the handling and experimental procedure. Mean plasma glucose levels were significantly suppressed at all time points during pregnancy (p<0.01) in relation to levels seen in age-matched control and lactating animals. In comparison, plasma insulin levels were elevated significantly during the later stages of gestation. Lactating animals showed a tendency towards lower basal insulin levels but this trend never achieved significance.

Plasma IR-GIP values were significantly elevated during lactation in comparison to pregnant values and on day 15 post partum the mean IR-GIP values of the lactating animals were significantly greater than both the control and pregnant animals. Control animals had IR-GIP values intermediate to the pregnant and lactating animals at all time points.
Figure 3.1.3  The Effects of Pregnancy and Lactation on Plasma Glucose, Plasma IR-Insulin and Plasma IR-GIP in Wistar Rats

Values are expressed as mean±SEM for groups of 12 (control) or 6 (pregnant or lactating) rats. Symbols (*, **) indicate values significantly different from the two other groups when assessed by one-way analysis of variance followed by Scheffe range test, *=p<0.05; **=p<0.01. Symbol (0) indicates a value significantly different from one other group at that time point when assessed by the same procedures, 0=p<0.05.
3.1.3.3 **Acute Nutrient Stimulation Tests**

Two acute nutrient stimulation tests were performed, an oral glucose tolerance test and an oral fat tolerance test.

3.1.3.3.1 **Oral Glucose Tolerance Test**

This test was performed on day 21 of pregnancy and again on day 16 of lactation. All plasma samples were assayed in a single assay and because the control values obtained at both study times were similar (mean values within 1 SD of each other) the control data has been pooled and is represented as one graph. The results are displayed graphically in Figure 3.1.4.

All three groups of animals gave clear responses to the oral glucose challenge. Fasting for 18 hours significantly lowered the plasma glucose concentration in the pregnant animals \((p<0.01)\). Repeated measures analysis of variance revealed differences between the groups \((p<0.01)\). These differences were shown to be between the lactating animals and the control animals, lactating animals having a superior glucose tolerance compared to the control animals. Pregnant animals had a similar tolerance to the control animals. Integrated glucose responses are given in Table 3.1.1, and confirm the improved glucose tolerance during lactation.
Figure 3.1.4  The Effects of Oral Administration of Glucose (51.2kJ/kg Body Weight) on Pregnant or Lactating Wistar Rats

Animals were fasted for 18 hours prior to the test. Values are expressed as mean±SEM for groups of 6 (pregnant and lactating) or 12 (control) rats. Symbols (**) indicate values significantly different from the two other groups when assessed by one-way analysis of variance followed by Scheffe range test. **=p<0.01. Symbols (0,00) indicate values significantly different from one other group when assessed by the same procedures, 0=p<0.05; 00=p<0.01.
Table 3.1.1 Integrated Plasma Glucose, IR-Insulin and IR-GIP Responses to an Oral Glucose Challenge (51.2kJ/kg Body Weight) in Pregnant or Lactating Wistar Rats

Values are expressed as mean±SEM for groups of 12 (control) or 6 (pregnant or lactating) rats. Symbol (*) indicates a value significantly different from the two other groups when assessed by means of one-way analysis of variance followed by Scheffe range test, *=p<0.05. Integrated responses were calculated as described in Section 2.9.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Integrated Plasma Glucose Response (mmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-Insulin Response (μmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-GIP Response (pmol/l).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Rats</td>
<td>188.5±17.5</td>
<td>53.4±10.4</td>
<td>5620.8±1377.2</td>
</tr>
<tr>
<td>Pregnant Rats</td>
<td>255.2±25.9*</td>
<td>142.1±25.9*</td>
<td>4748.3±1786.7</td>
</tr>
<tr>
<td>Lactating Rats</td>
<td>94.0±21.2*</td>
<td>46.1±7.7</td>
<td>6055.2±1628.8</td>
</tr>
</tbody>
</table>

The pregnant rats had an elevated fasting plasma insulin concentration in comparison to the other two groups (p<0.01). This elevated insulin concentration was further stimulated by the glucose challenge and the integrated insulin response of the pregnant animals (minus fasting values) was significantly greater than the response of the other two groups (Table 3.1.1). The lactating animals with their superior glucose tolerance did not show a modified insulin response.

Fasting plasma IR-GIP levels were similar between all three groups as were their responses to the glucose challenge.

3.1.3.3.2 Oral Fat Tolerance Test

This test was performed on the same day as the oral glucose tolerance using a different group of animals. The results are illustrated in Figure 3.1.5.
Figure 3.1.5  The Effects of Oral Administration of a Corn Oil Suspension (51.2kJ/kg Body Weight) on Pregnant or Lactating Wistar Rats

Animals were fasted for 18 hours prior to the test. Values are expressed as mean±SEM for groups of 6 (pregnant and lactating) or 12 (control) rats. Symbols (*, **) indicate values significantly different from the two other groups when assessed by one-way analysis of variance followed by Scheffe range test, *=p<0.05; **=p<0.01. Symbol 0 indicates a value significantly different from one other group when assessed by the same statistical procedures, 0=p<0.05.
An 18 hour fast significantly lowered plasma glucose concentration in pregnant rats (p<0.05). Oral fat administration had a modest yet significant lowering effect on the glucose level of the pregnant animals. Plasma IR-insulin concentrations were unaffected by the oral fat load. Differences between the three groups when assessed by repeated measures analysis of variance were identified by one-way analysis of variance to be due to the hyperinsulinaemia of the pregnant animals which was not modified by fasting or the test procedure. Fasting removed differences in basal IR-GIP levels between the groups. All groups gave a positive IR-GIP response to the fat load and there was no significant difference between the responses of the three groups.

3.1.3.4 Small Intestinal Entero-Endocrine Function in Pregnant and Lactating Wistar Rats

Hyperphagia of 7 days duration during pregnancy and of 17 days duration during lactation had significant effects on intestinal wet weight (Figure 3.1.6).

Figure 3.1.6 The Effects of Pregnancy and Lactation on Small Intestinal Wet Weight in Wistar Rats

Values are expressed as mean±SEM for groups of 6 (pregnant or lactating) or 12 (control) rats. Symbol (***) indicates a value significantly different from the two other groups when assessed by one-way analysis of variance and Scheffe range test. **=p<0.01.
Intestinal wet weights were not expressed on a body weight basis because the pregnant animals body weight reflects foetal weight and not the true weight of the dam. This is evident from the normalisation of the body weight of these animals so soon after parturition. The rank difference in mean intestinal weight (lactating>pregnant>control rats) reflected the degree of hyperphagia observed in the test groups.

The effect of pregnancy and lactation with concomitant hyperphagia on the content and the concentration of IR-GIP and IR-GLP-1(7-36)amide in the small intestine is shown in Figures 3.1.7 and 3.1.8.

Pregnancy with its concomitant hyperphagia had marked effects on the total content and concentration of IR-GIP in the small intestine. Lactation with its concomitant hyperphagia increased the total amount of IR-GIP in the small intestine to a similar extent as pregnancy but did not significantly alter the concentration of IR-GIP when increased wet weight of the small intestine was taken into account.

Again it was the pregnant animals which showed the greatest change in intestinal content and concentration of IR-GLP-1(7-36)amide. Intestines from pregnant and lactating animals had a higher extractable content of GLP-1(7-36)amide than control intestines (p<0.01). When wet weight of the intestine was taken into account the pregnant animals were the only group with an increased IR-GLP-1(7-36)amide concentration.
Figure 3.1.7 The Effects of Pregnancy and Lactation on Small Intestinal IR-GIP in Wistar Rats

Values are expressed as mean±SEM for groups of 12 (control) or 6 (pregnant or lactating) rats. Symbol (**) indicates a value significantly different from the two other groups when assessed by one-way analysis of variance followed by Scheffe range test, **=p<0.01. Symbol (00) indicates a value significantly different from control values when assessed by the same procedures, 00=p<0.01.
The Effects of Pregnancy and Lactation on Small Intestinal IR-GLP-1(7-36)amide in Wistar Rats

Values are expressed as mean±SEM for groups of 12 (control) or 6 (pregnant or lactating) rats. Symbol (**) indicates values significantly different from the two other groups when assessed by one-way analysis of variance followed by Scheffe range test, **=p<0.01. Symbols (0,00) indicate values significantly different from the control value when assessed by the same procedures, 0=p<0.05; 00=p<0.01.
3.1.4 Discussion

This study supports the observation of pregnancy and lactation being hyperphagic states. The hyperphagia associated with lactation was far greater than that seen during pregnancy, confirming previous reports (Campbell & Fell, 1964; Larralde et al., 1966; Craft, 1970; Cripps & Williams, 1975). In the present study, food consumption increased steadily throughout pregnancy, reaching a maximum level at 50% greater than the control rats intake. During lactation food intake peaked on day 16 post partum at approximately 260% greater than control rats. These results confirm the observations of Cripps & Williams (1975).

Basal measurements of glucose, IR-insulin and IR-GIP indicate pregnancy in rats to be a hyperinsulinaemic condition, with a corresponding decrease in plasma glucose. Similar findings have also been reported in some clinical studies (Hornnes et al., 1979). Lactating animals differed from control rats at the height of lactation only in their mean plasma IR-GIP levels, which reflected the degree of hyperphagia observed in these animals. It is interesting to note that with the severe hyperphagia present in these animals, the plasma glucose and IR-insulin levels were normal. This would suggest that milk production imposes a strong demand for glucose. Blood glucose levels have been reported to be lowered in lactation (Bliss et al., 1990) and this was proposed to be due to the increased nutrient needs for milk production and loss from the maternal body (Bumol et al., 1986). Plasma insulin has been reported to be decreased (Jones et al., 1984) and contrastingly increased (Stutter-Dub et al., 1974) during lactation. An elevated insulin concentration would make it difficult for the mammary gland to obtain sufficient glucose for milk production. The results of this study suggest that hyperphagia stimulates GIP release but because of the glucose drain of milk production insulin secretion is not augmented. Increased stimulation of the K cell has no long term effects and the degree of stimulation is still related to the amount of food consumed. The glucose challenge revealed no exhaustion or over stimulation.
of the K cell. The importance of the increased plasma IR-GIP concentration remains to be determined. Humans have been shown to secrete GIP in milk at a constant rate for the first six months of life (Berseth et al., 1990). Whether this is also true for rats remains to be established.

An 18 hour fast of the pregnant animals gave a milky appearance to subsequent plasma samples obtained during the acute studies. This is believed to be the result of the so called accelerated starvation of pregnancy (Herrera et al., 1969). The milky appearance is due to the raised plasma levels of triglycerides and ketone bodies which occur possibly as a means of sparing glucose and amino acids for foetal growth (Freinkel et al., 1971). This had no untoward effects on the assay procedures. Fasting the pregnant animals failed to reduce the insulin levels back to control values suggesting that the hyperinsulinaemia is of hormonal rather than dietary origin.

Lactating animals are very sensitive to food withdrawal. An 18 hour overnight fast leads to virtual complete inhibition of milk production (Munday & Williamson, 1981; Bussmann et al., 1984; Mercer & Williamson, 1986; Page & Kuhn, 1986). The physiological basis for this is not completely understood. Insulin is known to be involved in switching on lipogenesis and glucose extraction (Jones et al., 1984; Page & Kuhn, 1986). It has also been suggested that a gastrointestinal factor, a peptide, as yet unidentified but known to be insulin dependent in its actions may also be involved in switching on mammary glucose uptake (Page, 1989). The lactating animals were not therefore in a lactating state at the start of the acute tests. Whether the dose of glucose was sufficient to stimulate milk production can only be speculated. Experimentally, there was no option but to administer the oral challenge tests to fasted animals. When animals are hyperphagic to such a large degree there is a breakdown in their normal pattern of food intake, although a diurnal variation in milk secretion and food consumption is still present (Grigor & Thompson, 1987). It is therefore very difficult to oral dose an animal which already has a distended stomach due to its increased food
consumption. In addition, in such a small group of animals (n=6) individual variation in basal levels of glucose, IR-insulin and IR-GIP may well mask the response to the acute challenge. The lactating animals only differed from the control animals in their plasma glucose response to the oral glucose challenge. This indicates that the hyperphagia, even though severe, had no long term effects in these animals.

The oral glucose tolerance test revealed that glucose tolerance was not significantly improved in the pregnant state in spite of the prevailing hyperinsulinaemia, suggesting some degree of resistance to the actions of insulin. Insulin resistance has been well documented in clinical studies (Bleicher et al., 1964; Kühl, 1975; Hornnes et al., 1978). The pregnant animals had an exaggerated insulin response to the glucose load in comparison with the lactating and control rats. This increased insulin response to glucose in late pregnancy is well documented clinically (Lipson & Sharp, 1978).

Plasma IR-GIP responses to glucose or fat were not significantly modified by the states of pregnancy or lactation and basal levels were not significantly modified by pregnancy. Clinical studies in contrast have shown a moderately reduced secretion of GIP in response to acute glucose or mixed meal administration when compared to the post partum response (Hornnes et al., 1979; Jenssen et al., 1988). This may be a true species difference but this is unlikely however as the rat's digestive system has a very similar form and function to that of humans (Neil & Kesel, 1984). The enteroinsular axis of rats and humans also responds to nutrients in the same way and to a similar extent (Kikuoka et al., 1987). Pregnancy in humans is a time of moderately increased food intake (Durnin, 1987), not like the hyperphagia seen in rodents. Perhaps in the rat, the demands of an increased food intake cause sufficient stimulation of the K cell to override any other stimuli, possibly the insulin feedback, which would aim to reduce GIP secretion.
Conditions associated with hyperphagia have been shown to produce hypertrophic changes in the small intestine (Dowling, 1967). In lactation, the increased intestinal weight reported has been attributed to a secondary effect of the increased food intake (Campbell & Fell, 1964). The same is probably true in pregnancy. However, a contributory effect of hormonal change cannot be ruled out. Hypertrophy of all layers of the rat small intestine occurs during the later stages of pregnancy and lactation (Cripps & Williams, 1975). GIP and GLP-1(7-36)amide are both mucosal hormones. Changes in intestinal weight during pregnancy and lactation may not therefore bear a direct relationship to changes in mucosal hormone levels as the muscle layers of the intestine contribute to intestinal weight to a greater extent than changes in mucosa (Cripps & Williams, 1975). Both pregnancy and lactation produced a marked increase in intestinal IR-GIP and IR-GLP-1(7-36)amide content. This increase was not purely the result of hyperphagia as the increase was greater during pregnancy. When the hormonal content was expressed on a gram wet weight of intestine basis, the pregnant animals were the only group to show an increase in both IR-GIP and IR-GLP-1(7-36)amide.
3.2 The Ontogeny of Entero-Endocrine Small Intestinal Function

3.2.1 Introduction

In rats, as in all mammals, at birth there is an abrupt shift from the continuous, intravenous feeding of simple molecules, via the placenta, to an intermittent, enteral feeding regime which is necessarily of a more complex nature. This dietary change is accompanied by extensive functional and structural changes in the gastrointestinal tract (Henning, 1986) together with adjustments in intermediary metabolism (Duff & Snell, 1982; Jones & Rolph, 1985; Bassett, 1989). During intrauterine life, the foetus depends on glucose as its primary energy fuel. Amino acids are too valuable to catabolise for energy and fats (in the form of free fatty acids) are poorly transferred across the placenta. A newborn rat, as a consequence of this situation, has no white adipose tissue deposits and very low triglyceride storage in brown adipose tissue (Girard et al., 1973). The primary energy source of the neonatal rat at birth is glycogen. During suckling, the primary fuel of the neonate is fat. The majority of the fat being in the form of medium chain and long chain triglycerides (Grigor et al., 1986). At weaning, there is another abrupt nutritional change for the neonatal rat to contend with. The high fat milk diet is replaced by a high-carbohydrate, low-fat solid diet (laboratory diet composition was given in Section 2.1).

All these nutritional changes occur within a short space of time. The effects which these changes have on the entero-endocrine K and L cells of the rat intestine are unknown. The aim of this study was to monitor the small intestine and plasma, by means of precise RIA, for the two hormones GIP and GLP-1(7-36)amide to determine their ontogeny and to plot changes in their development with changes in nutritional status of the animal.
3.2.2 Methods

Fourteen Wistar albino time-mated, pregnant rats of an initial body weight of 200-250g, were utilised in this study. Small intestines were excised from foetal rats or neonates (on days 10, 14, 17, 19, 20, and 21 of intrauterine life and days 1, 2, 3, 7, 10, 14, 17, 20, 22, 23, 25, 31, 38, and 45 of life) and treated as described in Section 2.4.2. For the excision of foetal intestines dams were sedated with ether anaesthesia immediately prior to cervical dislocation. The dam was opened along the midline of the body and the womb exposed. The number of foetus was noted and each foetus was excised from its protective sac, wiped to remove amniotic fluid and the intestines exposed and excised. For very young foetuses intestines were excised as a whole (small and large intestine) due to poor discrimination of the two areas. Foetal rats were removed from a single mother at one time point unless the number of foetuses was less than six, when two or more dams would be sacrificed as appropriate. From day 14 post coitus it was also possible to obtain blood samples for plasma glucose, IR-insulin and from day 17 for IR-GIP assay. These samples were collected by cervical decapitation of the foetus and collection of blood in a heparinised Pasteur pipette. Plasma was treated as described in Section 2.4.1.

All dams were allowed to litter naturally (on day 22 post coitus) and litters were redistributed within 24 hours of birth to provide each dam with the normal size litter for this colony (8-10 offspring/dam). Neonates were sacrificed randomly and litters redistributed where necessary to maintain the normal litter size. Abrupt weaning was carried out on day 22 post partum. On this day offspring were sex separated, recaged in groups of 6 and provided with normal laboratory diet and water ad libitum (Section 2.1).
3.2.3 Results

Body weight and intestinal weight are given in Figures 3.2.1 and 3.2.2 respectively.

Figure 3.2.1 Body Weight During Late Foetal and Neonatal Life of Wistar Rats

Parturition occurred day 0. Abrupt weaning occurred day 22. Minus figures on the x axis indicate intrauterine life. Values are mean±SEM for a minimum of 6 animals at each time point.

Body weight gain showed a steep increase throughout the study.
Intestinal weight gain showed a sigmoidal development over time. This graph indicates that maximum rate of growth occurred within the first four weeks of life.

3.2.3.1 Ontogeny of Basal Plasma IR-GIP

In addition to IR-GIP, plasma glucose and IR-insulin were also assayed to provide additional information on plasma glucose homeostasis. A small amount of plasma could be obtained from day 14 of gestation onwards by decapitation of the foetus, but only from day 17 onwards was there sufficient for IR-GIP assay. Plasma glucose concentrations during late foetal and neonatal life are illustrated in Figure 3.2.3.
Repeated measures analysis of variance revealed highly significant changes in plasma glucose with time (p<0.01). Following parturition (day 0) there was a rapid and significant (p<0.01) fall in plasma glucose due to the removal of placental nutrition. Glucose levels started to rise over the first few days of life as lactation became established in the dams becoming significantly increased by day 3 of extrauterine life. During suckling, glucose levels were maintained at lower levels of normal than the levels seen after weaning (p<0.01 achieved on day 21 post-partum).
Figure 3.2.4 Plasma IR-Insulin During Late Foetal and Neonatal Life in Wistar Rats

Parturition occurred day 0. Abrupt weaning occurred day 22. Minus figures on the x axis indicate intrauterine life. Values are mean±SEM for a minimum of 5 animals at each time point. Symbol (**) indicates values significantly different from day -9 when assessed by Students unpaired t-test, **=p<0.01.

Plasma IR-insulin showed significant changes over time when assessed by repeated measures analysis of variance (p<0.01). There was a rapid rise in plasma IR-insulin from day 17-20 of intrauterine life. Insulin levels were significantly increased (p<0.01) from day 17-21 of intrauterine life compared with day 14. Maximum IR-insulin levels were seen on day 20 of foetal life. A second short-term rise in plasma IR-insulin was seen immediately after weaning, but this was not significant in comparison to the post weaning levels seen. Post-weaning basal IR-insulin levels were slightly higher than those seen during the suckling period.
Repeated measures analysis of variance revealed significant changes with time (p<0.01) in plasma IR-GIP concentration. Plasma IR-GIP levels started to rise prior to birth (a significant increase in plasma IR-GIP levels was seen from day 17 to day 19 of intrauterine life). Plasma IR-GIP levels increased at a more rapid rate over the first week of extrauterine life. Levels were significantly elevated during the suckling period from day 3 onwards. Basal IR-GIP levels were lower after weaning.

3.2.3.2 Ontogeny of Intestinal IR-GIP and IR-GLP-1(7-36)amide

Total extractable immunoreactive GIP and GLP-1(7-36)amide are illustrated in Figure 3.2.6.
**Figure 3.2.6** Small Intestinal IR-GIP and IR-GLP-1(7-36)amide Content in Late Foetal and Neonatal Wistar Rats

Parturition occurred day 0. Abrupt weaning occurred day 22. Minus figures on the x axis indicate intrauterine life. Values are mean±SEM for a minimum of 5 animals at each time point. Symbol (**) indicates a significant difference from day 20 when assessed by Student's unpaired t-test. **=p<0.01.

**Total Extractable IR-GIP**

![Graph showing Total Extractable IR-GIP over Days Postpartum.]

**Total Extractable IR-GLP-1(7-36)amide**

![Graph showing Total Extractable IR-GLP-1(7-36)amide over Days Postpartum.]

A repeated measures analysis of variance program revealed significant increases in both IR-GIP and IR-GLP-1(7-36)amide with time (p<0.01). IR-GIP and
IR-GLP-1(7-36)amide demonstrated a similar pattern of development in the small intestine. A steady increase in total intestinal content occurred over the first three weeks of extrauterine life. Large increases in both hormones occurred between days 20 and 22 of extrauterine life (p<0.01) corresponding to the weaning period. Following weaning there was a consistent increase in IR-GIP and IR-GLP-1(7-36)amide content.

**Figure 3.2.7** Small Intestinal IR-GIP and IR-GLP-1(7-36)amide Concentration in Foetal and Neonatal Wistar Rats

Parturition occurred day 0. Abrupt weaning occurred day 22. Minus figures on the x axis indicate intrauterine life. Values are mean±SEM for a minimum of 5 animals at each time point. Symbol (**) indicates p<0.01, when assessed by Student's unpaired t-test against values on day 17.
Significant changes in intestinal IR-GIP and IR-GLP-1(7-36)amide concentrations were seen over the ages studied when assessed by repeated measures analysis of variance (p<0.01). As with the plasma results, intestinal IR-GIP concentrations started to rise prior to birth; a significant increase was seen from day 17 - day 19 post coitus (p<0.01). The level continued to rise and maximum intestinal IR-GIP concentration was seen at day 1 of extrauterine life. Intestinal concentrations fell from day 1 of extrauterine life and were statistically indistinguishable from intrauterine levels by day 3 of extrauterine life. A second smaller and again transient decrease (p<0.01) followed by an increase (p<0.01) occurred at 10-14-17 days of extrauterine life.

GLP-1(7-36)amide concentration in the intestine was first detected above the sensitivity limits of the assay on day 17 of intrauterine life. Intestinal IR-GLP-1(7-36)amide concentration was significantly increased from day 17-21 of intrauterine life (p<0.01). Maximum intestinal IR-GLP-1(7-36)amide was seen on day 2 of extrauterine life. High IR-GLP-1(7-36)amide concentrations were maintained for the first seven days of extrauterine life.

3.2.4 Discussion

The fall in plasma glucose after birth has been reported previously (Gain & Watts, 1976). It is believed not to be serious if the birth is normal and the offspring are left in their normal environment with their mother. Plasma glucose during late intrauterine life was maintained in the presence of very high plasma insulin levels. In this study plasma insulin started to rise on day 19 of gestation and achieved its highest value on day 20. Similar results were reported by Schulze et al. (1977). It has been suggested that the foetal hyperinsulinaemia is necessary to increase protein synthesis in specific tissues (Johnson et al., 1990). The fact that plasma glucose is maintained while plasma insulin levels rise led Watts and colleagues to question whether foetal
insulin was in fact biologically active. Asplund (1972) has suggested that rats develop a glucose-sensitive type of insulin secretory mechanism during the first days of extrauterine life which is modulated by postnatal feeding. Later workers have reported that the foetal rat can respond to a glucose challenge by secreting insulin and the degree of insulin response increases as pregnancy proceeds (Kervran et al., 1979). This suggests that the intrauterine increases in both IR-GIP and IR-GLP-1(7-36)amide seen in this study could be involved in the rise in IR-insulin prior to birth.

The profile of basal plasma IR-GIP over the first few weeks of life has not previously been reported for rats. The reason for an increase in IR-GIP prior to birth which achieves basal levels normally seen in adult rats is unknown. If the K cell is being stimulated enterally than it can only be by the amniotic fluid which is believed to be swallowed to maintain electrolyte balance and to exercise the developing gastrointestinal tract. Foetal absorption of glucose from amniotic fluid has been described in rats at 19 days post coitus (Reusens et al., 1980). However, the nutrient absorption from amniotic fluid is believed to be negligible.

The increase of basal IR-GIP levels which are maintained during the suckling period are suggested to be the result of a high fat milk diet and the increased frequency of food consumption observed during the first 2-3 weeks of extrauterine life. A recent study in humans has shown that GIP is present in human milk at a concentration 25% of the normal basal plasma values (Berseth et al., 1990). Whether this is also true for rats remains to be established. The neonatal period is characterised by a lactose and fat rich diet containing triglycerides, galactose and glucose which in adults are known to stimulate the release of IR-GIP. Plasma IR-GIP levels had risen almost two fold by day 3 post partum from values at birth. It is possible that this delayed rise in IR-GIP is due to the establishment of lactation and the production of true milk. However Kühl et al. (1982) reported that neonatal pigs at 72 hours of age produced no significant GIP response to oral triglyceride infusion or oral glucose indicating an immaturity of the
entero-insular axis. Certainly by 6 days of age pre-term human babies have a functional GIP response to milk (Lucas et al., 1980). A study on full-term human infants has reported a functional entero-insular axis at 2 day of age (King et al., 1977). GIP has been shown to induce insulin secretion in 3 day old perfused rat pancreas (Bataille et al., 1977) suggesting that the entero-insular axis may well be functional at an early age in rats.

Intestinal weight increased maximally with extensive changes in digestive capacity and dietary composition at 3-4 weeks of life (Henning, 1986). Ontogeny and distribution of IR-GIP in rat small intestine was first reported by Gespach et al. (1979). In the present study, IR-GIP increased in the intestine prior to birth supporting the observed rise in plasma GIP and the development profile reported by Gespach and coworkers (1979). GIP secreting K cells have been observed by Moxey & Trier (1977) in the small intestine of human foetus at 12 weeks of gestation. The reason for this early development is unknown. The sharp peak in intestinal IR-GIP concentration observed on day 1 post partum has been reported to be due to an increase in duodenal GIP, which reaches maximum concentrations day 1 to day 14 post partum (Gespach et al., 1979). Gespach and fellow workers did not report any subsequent changes in concentration unlike that seen between days 14 and day 20 post partum in this study. The main increase in IR-GIP content of the small intestine occurred during the third week of life when adaptation from a milk to a solid diet was occurring. Ontogeny of IR-GIP appears to be related to fuel disposal over the first few weeks of life.

The ontogeny of IR-GLP-1(7-36)amide has only previously been inferred from the study of intestinal cell culture using non-specific glucagon antisera (Brubaker, 1987; Brubaker & Vranic, 1987). However, it was recently shown that mRNA for glucagon could be detected in the ileum during foetal life (Taylor et al., 1990; Jin et al., 1990). Taylor and coworkers reported mRNA was detectable from day 18 of gestation onwards and Jin and coworkers reported day 14 as the earliest time for detection.
Proglucagon synthesis was seen to proceed in the same way as GIP by Jin et al. (1990). In the present study, the development of IR-GLP-1(7-36)amide showed a similar pattern to that of GIP. Total content increased maximally at 3 weeks of age as with GIP. Interestingly IR-GLP-1(7-36)amide only became detectable at 20 days post coitus, and levels only rose significantly above basal following birth. Unlike GIP, IR-GLP-1(7-36)amide was then maintained at these elevated concentrations for the first week of life. The importance of these rapid increases in GIP and GLP-1(7-36)amide can only be speculated at. Certainly they are not stimulating insulin levels as the environment is normoglycaemic. However, as discussed in Sections 1.3.9 and 1.4.9, GIP and GLP-1(7-36)amide both have direct biological effects on white adipose tissue and since rats are born with very little adipose tissue, rapid deposition of fat is essential for future survival. Insulin cannot play a significant role in fat deposition at this time because of the need to stimulate glycogen breakdown and gluconeogenesis. Suckling is a time of net gluconeogenesis for rats. The importance of GIP and GLP-1(7-36)amide in stimulating adiposity may therefore be of greater importance at this time than their role in entero-insular communication. In suckling pigs, plasma levels of the two hormones have been shown to increase rapidly in fasted pigs allowed to suckle at the mother (Oben et al., 1991a). Both GIP and GLP-1(7-36)amide have been shown to directly stimulate fatty acid synthesis and incorporation, in a dose dependent manner, at different adipose sites (Oben et al., 1991c). Pigs are similar to rats in that they are born with very low triglyceride levels and no white adipose tissue deposits.

GLP-1 is a product of the intestinal L cell. Rats are born with an immature intestine. Enteroglucagon, another product of the L cell has been suggested by some to be a trophic factor in intestinal development (Jacobs et al., 1981; Watanabe et al., 1990). More recently it has been suggested to be an antitrophic factor (Gregor et al., 1990). The increase in intestinal GLP-1(7-36)amide and the maintenance of high levels during the first week of life may simply be due to control of small intestinal growth during this time period.
To conclude, plasma and intestinal GIP levels start to rise prior to birth as does intestinal IR-GLP-1(7-36)amide concentration, at a time when hyperinsulinaemia is present under basal glucose concentrations. Is this hyperinsulinaemia led by the foetus or is there some stimulus from the mother that promotes it? The importance of maternal diet to this hyperinsulinaemia was assessed by the following study.
3.3 Evaluation of the Origin of Foetal Hyperinsulinaemia

3.3.1 Introduction

Pilot investigations for the pregnancy and lactation study indicated that the removal of the mother's food supply 24 hours prior to parturition was not conducive to successful littering and raising of offspring. The ontogeny study revealed that around this time foetal rats become hyperinsulinaemic. It therefore raised the question whether the unsuccessful production of litters was a severe stress response of the dam, or possibly a more physiological problem, due to the interruption of the maternal nutrient supply, limiting final development of the foetus. The aim was to investigate whether the normal hyperinsulinaemia of late foetal life was affected by removal of the maternal diet.

3.3.2 Methods

Five, time-mated, pregnant rats were caged individually under normal experimental conditions on day 15 post coitus. Although this was a small number of rats, the numbers of offspring produced were 16 for the fed group and 23 for the fasted group. From day 17 of gestation, normal lab chow was replaced with a powdered diet comprising: 21.5% casein; 3.5% sunflower oil; 48% sucrose; 6.8% cellulose; 1% vitamin mixture; 4% mineral mixture; metabolisable energy 14MJ/kg. This was supplied to all rats ad libitum. Food intake was monitored daily. On day 19 food was removed from 3 of the rats for a period of 18 hours. On day 20 all rats were killed by cervical dislocation following light anaesthesia with ether. Foetuses were excised from the dam following exposure of the womb as described in Section 3.2.2. Body weight and liver weight of each foetus was noted and plasma collected for glucose, IR-insulin and IR-GIP determination as indicated in Section 2.4.1.
3.3.3 Results

Body weights of the pregnant rats over the 5 days of study showed a gradual increase over the period of food intake. Animals fasted 18 hours showed an average loss of 18±4g body weight (mean±SD) over the fasting period. Food intake was reduced in all 5 animals once transferred to the powdered diet (Figure 3.3.1).

Figure 3.3.1 Food Consumption of Pregnant Wistar Rats Before and After Transfer to a Powdered Diet

Values are mean±SEM for n=5 unless otherwise stated. The powdered diet was introduced on day 17. Three of the rats were fasted on day 19. Symbol (**) indicates a significant difference from day 17 when assessed by Student's paired t-test. **=p<0.01.

As can be seen the powdered diet was less acceptable to the rats than the pellet diet as mean food intake fell significantly on its introduction (p<0.01).
Figure 3.3.2  
Body Weight of Foetuses From Fed and 18 Hour Fasted Pregnant Wistar Rats

Values are mean±SEM. Foetuses were excised on day 20 of gestation from 5 pregnant rats; 2 in the fed state and 3 after an 18 hour fast. The number of foetuses is indicated on the graph.

No significant difference was observed in body weight between the two groups.

Figure 3.3.3  
Liver Weight of Foetuses From Fed and 18 Hour Fasted Pregnant Wistar Rats

Values are mean±SEM. Foetuses were excised on day 20 of gestation from 5 pregnant dams; 2 in the fed state and 3 after an 18 hour fast. The number of foetuses is indicated on the graph.

Liver weight was measured to indicate glycogen deposition; no significant difference was observed between the two groups.
Figure 3.3.4  Plasma Glucose, IR-Insulin and IR-GIP Concentration in Foetuses From Fed and 18 Hour Fasted Pregnant Wistar Rats

Values are mean±SEM. Foetuses were excised on day 20 of gestation from 5 pregnant rats: 2 in the fed state and 3 after an 18 hour fast. The number of foetuses is indicated on the graph.
Fasting the pregnant rats for 18 hours had no significant influence on foetal plasma glucose, IR-insulin or IR-GIP concentrations.

3.3.4 Discussion

Insulin has been implicated as a foetal growth factor. Growth of the foetus has been seen to occur at a rapid rate in late gestation (Battaglia & Meschia, 1986). Destruction of endogenous insulin by streptozotocin administration causes severe foetal growth retardation in lambs (Philipps et al., 1991). Transuterine insulin injection in rats at day 19 gestation has been shown to decrease protein accretion (synthesis) and thereby reduce turnover (Johnson et al., 1990). The hyperinsulinaemia of foetal rats is particularly effective at stimulating protein synthesis in brain, liver and heart (Johnson et al., 1990). The increase in plasma insulin in foetal rats correlates well with glycogen storage in the liver of the animals (Gain & Watts, 1976). Rats are born with very distended livers due to the glycogen stores being their only metabolic fuel to see them through the first hours of life. No difference in liver weight or any of the other parameters included in this study was observed.

The results of this small study indicate that short term removal of the maternal diet during late pregnancy has no untoward effects on foetal hyperinsulinaemia, the cause of which is unknown. The reason for the compromised littering of the dams in the pregnancy study must therefore have been a stress response of the mother, even though food was returned to them at least 24 hours prior to parturition.
3.4 The Influence of Over- and Undernutrition on IR-GIP and IR-GLP-1(7-36)amide Development in the Small Intestine of Neonatal Wistar Rats

3.4.1 Introduction

It was apparent from the ontogeny study reported in this Chapter (Section 3.2) that high plasma levels of IR-GIP persist during suckling in neonatal rats. These high levels of IR-GIP fail to augment insulin secretion due to the normal glucose levels present as the result of a high fat, low carbohydrate diet. Clinical studies have shown that bottle fed neonates have exaggerated IR-GIP and IR-insulin responses to nutrients when compared to breast fed infants (Lucas et al., 1980). It therefore remains to be answered whether this difference persists after weaning.

Manipulation of litter size is a procedure which has been utilised previously to look at enzyme induction during neonatal life (Duff & Snell, 1982). Alteration of litter size alters the body weight curve of neonates because of the difference in the degree of adiposity achieved by offspring rather than accelerating growth (Widdowson & McCance, 1961).

The aims of this study were to investigate whether over- and undernutrition would alter the intestinal development of GIP and GLP-1(7-36)amide. Additionally, the influence of altered neonatal nutrition on IR-GIP and IR-insulin responses to an oral glucose tolerance test were investigated, both during suckling and after a period on the weaned diet.
3.4.2 Methods

3.4.2.1 Animals and their Housing

Twelve pregnant, Wistar albino rats at 19-20 days of gestation were used in this study. The origin of these animals was discussed in Section 2.1. Rats were caged individually under normal experimental conditions (Section 2.1) and allowed to litter normally. All dams littered within 18 hours of each other. All offspring were removed within a short time after birth and randomly reallocated to mothers to provide the following sized litters:

1. Six litters where the number of offspring per dam was 4. These litters were considered to be overnourished.

2. Two litters where the number of offspring per litter was 10. These litters were considered to be normally nourished.

3. Three litters where the number of offspring per litter was 16. These litters were considered to be undernourished.

3.4.2.2 Experimental Procedure

Body weights of offspring were monitored on alternate days. On day 17 post partum, an oral glucose tolerance test (51.2kJ/kg body weight) was performed on 18 of the animals (6 rats from each treatment group). Animals were fasted for 18 hours prior to the test. The exact nature of the test was given in Section 2.2. Small intestines were collected from 6 offspring from each study group on the same day as described in Section 2.4.2. The remaining rats were redistributed within their original groups to maintain the litter sizes.
On day 22 post partum, the offspring were abruptly weaned. Animals were separated according to sex, caged in pairs and allowed access to normal laboratory diet and tap water ad libitum. On day 42 post partum, an oral glucose tolerance test was repeated on the same 18 animals. The dose of glucose used was the same, 51.2kJ/kg body weight. On conclusion of the test, animals were allowed access to food and after a period of 6 hours they were culled by cervical dislocation and their small intestines removed as described previously.

3.4.3 Results

3.4.3.1 Body Weight

Body weights are given for the 3 treatment groups over the period of study in Figure 3.4.1.

Figure 3.4.1 The Effects of Over- and Undernutrition During the Suckling Period on the Body Weight of Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Abrupt weaning occurred day 22 post partum. Symbols (*, **) indicate the first time point at which the body weights of pups from the various litters became significantly different when assessed by one-way analysis of variance and Duncan's range test, *=p<0.05; **=p<0.01.
The body weights of pups in the 3 study groups became significantly different from day 3 post partum onwards (overnourished>normal>undernourished). These differences persisted even after weaning onto normal laboratory diet. Following weaning, food intake between the three study groups was similar.

3.4.3.2 Oral Glucose Tolerance Test

The two tests at 17 days and 42 days post partum will be considered together (Figures 3.4.2, 3.4.3, 3.4.4).

Analysis over time revealed that at 17 days of age all three study groups had a significantly superior glucose tolerance when compared with their response at 42 days of age (P<0.01). Both the over- and undernourished groups had a lower fasting plasma glucose value when compared with the control group of normally nourished animals. As a result of their lower fasting values, their maximum glucose concentration at 30 minutes was also lower. Incrementally the rise in plasma glucose over the first 30 minutes was similar for all three groups at 17 days of age (Table 3.4.1).
The Effects of Over- and Undernutrition During the Neonatal Period on the Plasma Glucose Response to an Oral Glucose Tolerance Test (51.2kJ/kg body weight)

Animals were fasted 18 hours prior to the test. Values are expressed as mean±SEM for groups of 6 rats. Symbol (**) indicates significant differences between two of the three groups when assessed by one-way analysis of variance and Duncan's range test, **=p<0.01.

17 Days of Age

42 Days of Age
Table 3.4.1 Incremental Plasma Glucose Response to an Oral Glucose Tolerance Test (51.2kJ/kg body weight) in Over- and Undernourished Neonatal Wistar Rats at 17 Days of Age

Values are expressed as mean±SEM for groups of 6 animals. The incremental response was calculated as described in Section 2.9.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Incremental (0-30 minutes) Plasma Glucose Response mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Over Nourished Rats</td>
<td>5.17 ± 0.53</td>
</tr>
<tr>
<td>Control Normally Nourished Rats</td>
<td>5.20 ± 0.28</td>
</tr>
<tr>
<td>Under Nourished Rats</td>
<td>5.52 ± 0.99</td>
</tr>
</tbody>
</table>

At 42 days of age there was no significant difference between any of the three groups at any time point.

The improved glucose tolerance during suckling was not accompanied by an increased insulin response (Figure 3.4.3). At 17 days of age the overnourished animals had significantly higher fasting plasma insulin values than the undernourished animals and this difference was also present 120 minutes after the glucose administration. Fasting plasma insulin levels were significantly higher at 17 days of age, compared with 42 days of age for all groups, (p<0.05).

The undernourished animals actually gave a significantly lower plasma IR-insulin response at 17 days of age (compared with 42 days of age), despite improvement of glucose tolerance (p<0.01).
The Effects of Over- and Undernutrition During the Neonatal Period on the Plasma IR-Insulin Response to an Oral Glucose Tolerance Test (51.2kJ/kg body weight)

Animals were fasted 18 hours prior to the test. Values are expressed as mean±SEM for groups of 6 rats. Symbol 0, indicates a significant difference between two of the three groups when assessed by one-way analysis of variance and Duncan's range test, 0=p<0.05.

17 Days of Age

42 Days of Age
Figure 3.4.4 The Effects of Over- and Undernutrition During the Neonatal Period on the Plasma IR-GIP Response to an Oral Glucose Tolerance Test (51.2kJ/kg body weight)

Animals were fasted 18 hours prior to the test. Values are expressed as mean±SEM for groups of 6 rats. Symbols (○○) indicate significant differences between two of the three groups when assessed by one-way analysis of variance and Duncan’s range test, ○○=p<0.01.

17 Days of Age

Fasting plasma IR-GIP levels were elevated at 17 days of age in all groups (p<0.01). Repeated measures analysis of variance revealed differences in the plasma

42 Days of Age

Page 122
IR-GIP response of the three groups at 17 days. This difference was identified by one-way analysis and range tests to be due to the exaggerated IR-GIP response of the overnourished animals. Fasting plasma IR-GIP concentrations and the acute IR-GIP responses to the glucose load were similar for all three groups on day 42.

3.4.3.3 Intestinal Measurements of IR-GIP and IR-GLP-1(7-36)amide

Intestinal wet weight measurements of the three treatment groups are summarized in Figure 3.4.5, and total intestinal IR-GIP content is summarized in Figure 3.4.6.

No significant differences were present in intestinal weight between the three groups at either 17 or 42 days of age.
Figure 3.4.5 The Effects of Neonatal Over- and Undernutrition on Intestinal Wet Weight in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats.

17 Days of Age

42 Days of Age
The Effects of Neonatal Over- and Undernutrition on Total Intestinal IR-GIP Content in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbols (*, **) indicate values significantly different from the other two groups when assessed by means of one-way analysis of variance and Duncan's range test, *=p<0.05; **=p<0.01.

17 Days of Age

Total extractable IR-GIP was significantly greater in the overnourished animals at 17 days of age (p<0.01). By 42 days of age this difference was reversed and total IR-GIP content was significantly lower in the overnourished rats (p<0.05).
The relative concentration of intestinal IR-GIP is shown in Figure 3.4.7.

**Figure 3.4.7**  The Effects of Neonatal Over- and Undernutrition on Intestinal IR-GIP Concentration in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbol (*) indicates a value significantly different from the other two groups when assessed by means of one-way analysis of variance and Duncan's range test, * = p<0.05.

**17 Days of Age**

![Graph showing IR-GIP concentration at 17 days of age](image)

**42 Days of Age**

![Graph showing IR-GIP concentration at 42 days of age](image)
When wet weight of the intestine was used to give the relative concentration of intestinal IR-GIP, the overnourished rats had a higher concentration of intestinal IR-GIP at 17 days (p<0.05). By 42 days of age this difference was no longer apparent. Intestinal GLP-1(7-36)amide can be considered in the same way.

**Figure 3.4.8** The Effects of Neonatal Over- and Undernutrition on Total Intestinal IR-GLP-1(7-36)amide Content in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbol (φ) indicates a value significantly different from the normal group when assessed by means of one-way analysis of variance and Duncan's range test, φ=p<0.05.

**17 Days of Age**

- ■ Over nourished
- □ Normal
- ☐ Under nourished

**42 Days of Age**

- ■ Over nourished
- □ Normal
- ☐ Under nourished
At 17 days of age total intestinal GLP-1(7-36)amide content was increased in both the over- and undernourished groups (p<0.05). By 42 days of age the three groups had similar total IR-GLP-1(7-36)amide levels.

**Figure 3.4.9** The Effects of Neonatal Over- and Undernutrition on Intestinal IR-GLP-1(7-36)amide Concentration in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbol (○) indicates a value significantly different from the normal group when assessed by means of one-way analysis of variance and Duncan's range test, ○=p<0.05. Symbol (*) indicates a value significantly different from the two other groups when assessed by the same methods of analysis, *=p<0.05.

**17 Days of Age**

- ● Over nourished
- ○ Normal
- □ Under nourished

**42 Days of Age**

- ● Over nourished
- ○ Normal
- □ Under nourished
At 17 days of age intestinal IR-GLP-1(7-36)amide concentrations were significantly increased in both the overnourished and undernourished groups. By day 42, the concentration of GLP-1(7-36)amide had increased in normal rats beyond that of the overnourished rats but the undernourished rats still had a significantly higher GLP-1(7-36)amide concentration when compared with the overnourished rats.

3.4.4 Discussion

From about 14 days of age, neonates become more active and towards weaning they start to nibble at the laboratory chow provided for the dam. One possible reason for this may be the decline in food intake by the mother resulting in reduced milk production. As a result of this, the oral glucose tolerance test was performed at 17 days of age rather than just prior to weaning of the animals. During the suckling period, plasma IR-GIP was elevated both when fasted and in response to an acute glucose challenge for all groups. Glucose tolerance was improved. However, in spite of the prevailing modest hyperglycaemia and elevated IR-GIP levels, the insulin response was not augmented beyond that seen in the weaned animal. Intestinal IR-GIP was elevated only in the overnourished animals during suckling, as was IR-GLP-1(7-36)amide. By 42 days of age, all measured parameters were normalised in the overnourished animals. It can therefore be concluded that overnutrition during the neonatal period transiently augments the function of the endocrine GIP secreting cell. The elevated fasting IR-GIP level is presumed to be due to the high fat diet consumed by the offspring. Milk contains triglycerides, glucose and galactose (in the form of lactose), nutrients all known to stimulate GIP secretion in adults. Undernutrition had no effect on intestinal IR-GIP concentrations. It did, however, result in elevated intestinal IR-GLP-1(7-36)amide concentrations by 17 days of age. These results were not normalised 21 days after weaning.
The observation that insulin secretion was not increased during suckling but glucose tolerance was improved has also been reported in human neonates (King et al., 1977). The results suggest that the increased plasma IR-GIP concentration is not augmenting insulin secretion at this time, in spite of the moderate hyperglycaemia present. The importance of the biological roles of GIP and GLP-1(7-36)amide in white adipose tissue stimulation have been suggested previously (Section 3.2.4). The newborn rat has 0.5-1% body fat at birth and this increases to 8-12% by 18 days of age (Haggarty et al., 1987). The importance of GIP and GLP-1(7-36)amide in fat deposition is at present unknown, it can be suggested that the action of these hormones on fat metabolism may be important at this stage of neonatal development.

Undernutrition of rats resulted in a lower rate of body weight gain. To what extent malnutrition could have been present in the rats cannot be evaluated. The distal small intestine is however very sensitive to malnutrition (Hamilton et al., 1983) and rats are born with an under developed gastrointestinal tract (Henning, 1986). Enteroglucagon, another product of the GLP-1(7-36)amide secreting cell, has been suggested to be a trophic factor for intestinal development (Jacobs et al., 1981; Watanabe et al., 1990). More recently enteroglucagon has been suggested to be an antitrophic hormone (Gregor et al., 1990). The increase in GLP-1(7-36)amide in undernourished animals may therefore have no specific role itself and may be a consequence of requirements for other hormones sequenced on the same proglucagon molecule.
3.5 The Effects of Cold Acclimation, with and without Concomitant Hyperphagia, upon Basal and Nutrient Stimulated Plasma IR-GIP and Intestinal IR-GIP and IR-GLP-1(7-36)amide

3.5.1 Introduction

Animals exposed to low environmental temperatures (experimentally usually 5°C) demonstrate increased metabolism in order to maintain body temperature (Jansky, 1973). Initially shivering thermogenesis is the primary means of heat production. With prolonged cold exposure (greater than three weeks), non-shivering thermogenic mechanisms (primarily due to brown adipose tissue metabolism) increase in predominance and ultimately replace shivering to maintain heat production (Nedergaard et al., 1982). This adaptive process is known as cold acclimation. The changes in vascularity and appearance of brown adipose tissue (BAT) which occur during cold acclimation have been fully reviewed by Smith & Horwitz (1969) using rat interscapular BAT. Activation of BAT is considered to result from increased adrenergic activity during cold exposure (Helle et al., 1980).

In order to meet the increased energy demands of cold stimulated thermogenesis, hyperphagia occurs to avoid compromising body weight gain too severely (Cottle & Carlson, 1954). In 1980, Rothwell & Stock suggested that prolonged hyperphagia may produce an adaptation in the dietary induced thermogenic response. It was proposed that animals were capable of increasing their ability to respond calorigenically to noradrenaline. On the basis of this, it has been suggested that cold exposure and hyperphagia could be synergistic in the development of cold adaptability, possibly through activation of the sympathoadrenal system (Tulp et al., 1982). Prevention of hyperphagia, during cold adaptation, by restricted feeding has produced conflicting results. After two weeks cold exposure, Johnson et al. (1982) reported that pair feeding (with warm control rats) limited BAT hypertrophy (despite
similar sympathetic innervation). Other research groups, using a three week study period, have reported no influence of pair feeding on BAT hypertrophy (Kuroshima & Yahata, 1985; Puerta & Abelenda, 1987). BAT primarily utilises free fatty acids as its main metabolic substrate. These free fatty acids are imported from white adipose tissue and also obtained directly from lipolysis liberating free fatty acids stored within BAT. Restricted feeding would perceivably limit substrate availability to BAT. The reported lack of influence of restricted feeding on BAT hypertrophy in some studies has led to suggestions that under these conditions ketone bodies may form part of the substrate for BAT (Wright & Agius, 1983).

As stated previously, BAT is activated by increased adrenergic activity during cold exposure. Fasting (48 hours) however, reduces the responsiveness of the sympathetic nervous system and the adrenal medulla to acute cold exposure (Avakian & Horvath, 1981). Therefore, other components are involved in activation of BAT in cold environments. Glucagon has been implicated as a potent stimulator of BAT in cold exposed rats (Howland, 1986). Increased plasma glucagon concentrations have been reported by several research groups during cold exposure (Kuroshima et al., 1978; Edwards & Howland, 1986). Correspondingly cold acclimation is known to increase glucose turnover and to lower plasma insulin concentrations by as much as 50% (Beck et al., 1967; Vallerand et al., 1983). This fall in insulin levels is sufficient to reverse the hyperinsulinaemic, glucose intolerant effects of a hyperphagic cafeteria diet (Vallerand et al., 1986). The increase in plasma glucagon therefore results from a decrease in insulin secretion removing the suppressive action of insulin on the A cells of the pancreas, (Curry & Curry, 1970; Edwards & Howland, 1986). The fall in plasma insulin has been reported to be due to acute cold exposure as it is rapidly abolished by re-exposure to a warm environment (Vallerand et al., 1983). Other research groups disagree, reporting slow and progressive sequential changes in insulin levels and thus implicating cold acclimation to be involved in the fall of plasma insulin concentrations (Edwards & Howland, 1986).
The effects of cold acclimation on glucose homeostasis and insulin secretion are therefore well documented. The possible interactions of cold acclimation and hyperphagic effects on the entero-insular axis have not been considered. This study proposed to investigate this area of research by monitoring plasma IR-GIP concentrations during the period of cold acclimation and intestinal IR-GIP and IR-GLP-1(7-36)amide after six weeks of cold acclimation. The protocol involved two cold exposed study groups; one food restricted (to the level of intake of the thermoneutral control age matched rats) and one allowed to feed ad libitum. This procedure allowed monitoring of the influences of hyperphagia induced by cold exposure and the effects of cold exposure not attributable to hyperphagia on entero-endocrine function.

3.5.2 Methods

3.5.2.1 Animals and their Treatment

Thirty male, Wistar albino rats (180-190g body weight) were utilised. The origin and handling of the animals is discussed in Section 2.1. The rats were maintained for one week at 22±2°C under normal experimental conditions, caged in groups of 3 or 4 (Section 2.1). This time allowed the rats to become familiar with the procedures of handling, weighing etc. During this week, one group of rats per treatment were randomly selected and bled to obtain basal measurements for plasma glucose, IR-insulin and IR-GIP as described in Section 2.4.1. On day 7, the animals were allocated to one of the following treatment regimes:
1. Thermoneutral exposure (22±2°C) with *ad libitum* feeding, two groups of six animals.

2. Cold exposure (4±1°C) with *ad libitum* feeding, two groups of seven animals.

3. Cold exposure (4±1°C) with food restricted to the level of consumption of thermoneutral control rats, one group of seven animals.

The rats were caged in groups of 3 or 4 with the aim of reducing the initial stress of cold exposure. The nature of the diet provided throughout the study is described in Section 2.1. All animals were allowed free access to tap water, and a standard 12 hour photoperiod (0700-1900 hours light) was maintained for both warm and cold treated animals. In view of the reported inter-relationship between feeding pattern and endogenous lipogenesis/lipolysis cycle in rats (Le Mangen, 1983) cold exposed pair fed animals were provided at 1600 hours daily with an amount of food eaten by thermoneutral control rats on the previous day. The pair-fed rats rapidly consumed the food provided. All experimental procedures were performed at 0900-1200 hours.

3.5.2.2 Experimental Procedures

Body weight and food intake were monitored daily as described in Section 2.3. This allowed the opportunity of regularly inspecting the health of the animals. Plasma samples for the basal measurements of plasma glucose, IR-insulin and IR-GIP were obtained at weekly intervals, from all three treatment groups, as described in Section 2.4.1.

Acute nutrient response tests were performed 14 days and 35 days after the start of cold exposure (i.e. during and after completion of the process of cold acclimation).
following an 18 hour fast of the rats. Pilot studies, using non-fasted animals so as not to interfere with the process of cold acclimation, revealed problems with the dosing of hyperphagic animals. Individual animal variation in response to the test under these conditions was so great that interpretation of the results was difficult. Pair fed rats could not be fasted for 18 hours without altering their feeding time and so they were excluded from the acute tests. The acute challenges utilised were an oral glucose tolerance test (51.2 kJ/kg body weight) and an oral fat tolerance test (51.2 kJ/kg body weight). The exact nature of these tests is described in Section 2.2.

An insulin sensitivity test was performed on day 42 of cold acclimation using porcine insulin (Actrapid MC, Novo Industria, Copenhagen), at a dose of (1U/2ml) per kg body weight, diluted in 0.9% (w/v) saline and adjusted to pH 3.5.

At the end of the experiment (day 42), small intestines were isolated from the rats and treated in the manner described in Section 2.4.2. In addition the interscapular fat pad of each animal was removed and the BAT carefully dissected from adherent white adipose tissue and skeletal muscle. The wet weight of interscapular BAT was subsequently determined.

3.5.3 Results

3.5.3.1 Body Weight and Food Intake

Daily food intake for the three study groups is depicted graphically in Figure 3.5.1.
Figure 3.5.1 Food Consumption of the Three Study Groups Demonstrating the Extent of Hyperphagia in Cold Exposed Ad Libitum Fed Wistar Rats

Values are expressed as mean±SEM for groups of 6 (warm control), 7 (cold ad libitum fed) and 7 (cold pair fed) rats. Animals were exposed to the cold from day 7 onwards. Symbol (**) indicates values significantly different from the other two study groups at that time point and subsequent time points when assessed by one-way analysis of variance and Duncan's multiple range test. ** = p<0.01.

Repeated measures analysis of variance revealed a significant effect of environmental temperature on feeding pattern (p<0.01). One-way analysis of variance and range testing revealed that the cold ad libitum fed rats became hyperphagic (p<0.01) on day 13 of the study (6 days after cold exposure began). Food intake in these rats increased over the following 24 hours and then remained relatively stable at approximately 1.5 times greater than normal intake for the duration of the study.

Dietary chow stored at 5°C gained weight due to water adsorption from the damp environment at a rate of 10g/100g food over a 24 hour period. This weight gain was not compensated for in the experimental procedure as it only affected the cold ad libitum fed group. All food in the hopper of the cages was removed and replaced with fresh chow every 24 hours to ensure the continuing acceptability of the diet and to
prevent mould spoilage. As a consequence of this water adsorption the increased food intake following cold exposure was probably underestimated.

**Figure 3.5.2**  
The Effects of Cold Acclimation With and Without Concomitant Hyperphagia on the Body Weight of Wistar Rats

Values are expressed as mean±SEM for groups of 6 (warm control) or 7 (cold acclimated *ad libitum* fed) and 7 (cold pair fed) rats. Animals were exposed to the cold from day 7 onwards. Symbols (**) indicate values significantly different from the other two groups at that time point when assessed by one-way analysis of variance and Duncan’s multiple range test, **=p<0.01.

Body weights significantly increased during the 6 week study in all three groups as indicated by repeated measures analysis of variance (p<0.001). The rate of growth correlated in each of the three groups not only with the feeding regime but also with the environmental temperature. One-way analysis of variance followed by Duncan’s range test revealed significant differences in the body weight of the cold exposed *ad libitum* fed group and the warm control animals after 6 days of cold exposure. This difference did not persist; two days after the body weight difference became significant, the cold exposed animals became hyperphagic and exhibited *catch up growth* to the extent that differences in mean body weight were no longer significant by day 15 of the study.
The cold pair fed animals surprisingly never lost weight. They continued to develop but at a slower pace than the other two groups. Their mean body weight was decreased ($p<0.01$) compared with the other two groups after 9 days of cold exposure (day 16 of the study). Body weight of this group remained significantly depressed from that point onwards.

3.5.3.2 Basal Plasma Glucose, IR-Insulin and IR-GIP during the process of Cold Acclimation

The mean results of basal plasma glucose, IR-insulin and IR-GIP values are shown in Figure 3.5.3.
Figure 3.5.3 The Effects of Cold Acclimation With and Without Concomitant Hyperphagia on Plasma Glucose, IR-Insulin and IR-GIP

Values are expressed as mean ±SEM for groups of 6 (warm control) or 7 (cold ad libitum fed) and 7 (cold pair fed) rats. Symbols (*, **) indicate values significantly different from the other two study groups when assessed by one-way analysis of variance followed by Duncan's multiple range test *=p<0.05; **=p<0.01. Symbols (0, 00) indicate values significantly different from one other study group when assessed by the same methods of analysis, 0=p<0.05; 00=p<0.01.

- Control Rats
- Cold Ad Libitum Fed Rats
- Cold Pair Fed Rats
Repeated measures analysis of variance revealed significant differences in plasma glucose concentration with environmental temperature \((P<0.01)\), but no significant influence of feeding regime. Upon exposure to the cold, plasma glucose fell slightly in both cold treated groups. This fall became significant in pair fed animals after 10 days of cold exposure (day 17 of the study) but was never below the normal basal range for rats. As cold acclimation progressed plasma glucose concentrations of both cold treated groups became significantly elevated in comparison to warm control rats. Values remained elevated until the conclusion of the study.

Both environmental temperature and feeding regime significantly affected basal plasma insulin concentrations over time \((p<0.05 \text{ and } p<0.001, \text{ respectively})\). Both cold exposed treatment groups demonstrated a significant initial fall in plasma insulin levels after 3 days of cold exposure \((p<0.01)\). In the cold \textit{ad libitum} fed animals, this was followed by a return to increased insulin levels comparable with those seen in the warm control animals. Pair-fed animals, however, had reduced basal insulin levels throughout the study \((p<0.01)\).

Basal plasma IR-GIP levels were significantly influenced by environmental temperature and feeding regimen over the time course of the study \((p<0.05 \text{ and } p<0.01, \text{ respectively})\). One-way analysis of variance and range testing indicated a significant increase in plasma IR-GIP in the cold \textit{ad libitum} fed animals in comparison to the cold pair-fed animals \((p<0.01)\) on days 17 and 23 of the study (i.e. 10 and 15 days after cold exposure was initiated). The raised plasma IR-GIP levels of the cold \textit{ad libitum} fed group then fell back to levels comparable with the pair-fed group on days 30 and 38. The warm control animals had IR-GIP concentrations intermediate to the other two groups until day 30. At this time, the pair-fed animals had suppressed IR-GIP levels in comparison with the warm controls \((p<0.05)\). By day 38 of the study, no significant differences were present between any of the three groups.
3.5.3.3 **Acute Oral Nutrient Stimulation during the process of Cold Acclimation; Effects on Plasma Glucose, IR-Insulin and IR-GIP**

3.5.3.3.1 **Oral Glucose Tolerance Test**

This test was performed on day 21 and again on day 42 of the study, (i.e. at 2 and 5 weeks of cold acclimation). The two tests will be considered together in order to illustrate differences due to the on-going process of cold acclimation. All plasma samples were analysed in a single assay and because control values obtained at 21 and 42 days were similar (mean values within one standard deviation of each other) the control data have been pooled and are represented as one graph (Figure 3.5.4).
Figure 3.5.4 The Effects of the Process of Cold Acclimation on the Response to an Acute Oral Glucose Challenge (51.2kJ/kg body weight)

Animals were fasted for 18 hours prior to the test. Values are expressed as mean±SEM for groups of 12 (warm control) or 7 (cold acclimated ad libitum fed) or 7 (cold acclimated pair fed) rats. Symbol (*) indicates a value significantly different from the two other treatment groups at that time point when assessed by one-way analysis of variance followed by Scheffe range test, *=p<0.05. Symbol 0 indicates a value significantly different from one other group at that time point when assessed by the same methods of analysis, 0=p<0.05.
Plasma glucose concentration differences between the three groups were overcome by an 18 hour fast. Repeated measures analysis of variance revealed that the differences between the groups over time were significant (p<0.01), whilst the differences within the groups were not. Both of the cold acclimated groups had a superior glucose tolerance at 30 minutes compared with the control animals (p<0.05). By 60 minutes this difference persisted between the 2 week cold acclimated and warm control rats only. The 5 week cold exposed animals had an intermediate tolerance to glucose at this time. Integrated glucose, IR-insulin and IR-GIP responses are given in Table 3.5.1. These confirm the significantly superior handling of the glucose challenge by the cold acclimated animals.

Table 3.5.1 Integrated Plasma Glucose, IR-Insulin and IR-GIP Responses to an Oral Glucose Challenge in Wistar Rats Undergoing Cold Acclimation

Values are expressed as mean±SEM for groups of 7 (cold acclimated) or 12 (warm control) rats. Symbols (*, **) indicate values significantly different from the control group when assessed by one way analysis of variance followed by Scheffe range test, *=p<0.05; **=p<0.01. Integrated responses were calculated as described in Section 2.9.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Integrated Plasma Glucose Response (mmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-Insulin Response (ng/ml).hr⁻¹</th>
<th>Integrated Plasma IR-GIP Response (pmol/l).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm Control Rats</td>
<td>176.3 ± 15.4</td>
<td>91.8 ± 12.8</td>
<td>3579.2 ± 99.3</td>
</tr>
<tr>
<td>2 Weeks Cold Exposed Rats</td>
<td>70.7 ± 16.0 **</td>
<td>129.9 ± 28.2</td>
<td>8203.8± 104.8 *</td>
</tr>
<tr>
<td>5 Weeks Cold Exposed Rats</td>
<td>95.3 ± 25.4</td>
<td>69.3 ± 21.1</td>
<td>4657.0 ± 98.2</td>
</tr>
</tbody>
</table>

Mean fasting plasma IR-insulin concentrations were significantly lower at both stages of cold acclimation (p<0.05). Repeated measures analysis of variance revealed significant response differences between groups (p<0.05). However, within the groups the response over time was similar. The plasma IR-insulin response after 2
weeks of cold acclimation was similar to the response of warm control rats. By 5 weeks of cold acclimation, the maximum plasma insulin concentration attained was significantly reduced in comparison to the warm control animals. The overall quantity of insulin secreted (assessed from the integrated value in Table 3.5.1) in response to the glucose challenge was not significantly modified in spite of this.

Differences in basal IR-GIP values were no longer apparent after fasting the animals for 18 hours. The plasma IR-GIP response to the glucose challenge was significantly different between the groups over time (p<0.05). These differences were revealed to be between the warm control rats and the 2 week cold exposed rats. The two week cold exposed rats demonstrated a heightened IR-GIP response to the glucose challenge at the 60 minute time point and an augmented integrated response (p<0.05, Table 3.5.1). Both cold acclimated groups showed a slower return to base line values when compared with warm control rats.

An oral glucose challenge during cold acclimation produced a superior glucose tolerance which was maintained as cold acclimation proceeded. The superior glucose tolerance was not due to a modified insulin response but was accompanied by an augmented IR-GIP response during the early stages of cold acclimation.

3.5.3.3.2 Oral Fat Tolerance Test

Again this test was performed after 14 and 35 days of cold exposure in order to investigate tolerance during and after complete cold acclimation. Different animals from the same groups as previously investigated were used (Figure 3.5.5).
Figure 3.5.5  The Effects of the Process of Cold Acclimation on the Response to an Acute Oral Fat Challenge (51.2k J/kg Body Weight)

Animals were fasted 18 hours prior to the test. Values are expressed as mean±SEM for groups of 12 (warm control) or 7 (cold acclimated) rats. Symbol 0 indicates a value significantly different from the control value at that time point when assessed by a one-way analysis of variance followed by Scheffe range test, 0=p<0.05.
Plasma glucose and plasma IR-insulin concentrations showed no significant response to the oral fat challenge, either between or within each of the 3 study groups, when assessed by repeated measures analysis of variance. Plasma IR-GIP response likewise showed no difference in response between groups or within each group when assessed over time. When single time points were analysed, the rats which had been subjected to 2 weeks of cold exposure had a significantly greater (p<0.05) maximum IR-GIP response at 60 minutes when compared with warm control animals. By 5 weeks of cold acclimation, this difference had disappeared. Integrated IR-GIP responses however revealed that this difference in maximum IR-GIP response had no significant effect on the overall IR-GIP response (Table 3.5.2).

Table 3.5.2 Integrated Plasma IR-GIP Response to an Oral Fat Challenge in Wistar Rats Undergoing Cold Acclimation

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Integrated Plasma IR-GIP Response (pmol/l).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm Control Rats</td>
<td>1328.4 ± 676.7</td>
</tr>
<tr>
<td>2 Weeks Cold Exposed Rats</td>
<td>1617.2 ± 565.2</td>
</tr>
<tr>
<td>5 Weeks Cold Exposed Rats</td>
<td>1533.6 ± 1621.6</td>
</tr>
</tbody>
</table>

3.5.3.4 Insulin Sensitivity Test

This intraperitoneal challenge was performed after 42 days of cold acclimation in 18 hour fasted animals (Figure 3.5.6).
The Effects of Six Weeks Cold Acclimation on Insulin Sensitivity in Wistar Rats

Porcine insulin was administered in a single intraperitoneal injection at time 0 at a dose of (1U/2ml)/kg body weight. Values are expressed as mean±SEM for 7 (cold acclimated) or 6 (warm acclimated) rats. Symbols (*, **) indicate values significantly different from control values at that time point when assessed by Student's unpaired t-test, *=p<0.05; **=p<0.01.

Repeated measures analysis of variance revealed significant differences in plasma glucose concentration between the two groups over time (p<0.01) with an interaction of environmental temperature. Fasting insulin values of the two groups were similar but over the following 60 minutes of the test, the cold acclimated animals had superior insulin sensitivities as shown by the steeper fall in plasma glucose concentration. Cold acclimated animals did however show a return to control plasma glucose concentrations 120 minutes after insulin administration.
The Effects of Cold Acclimation with and without Concomitant Hyperphagia on Brown Adipose Tissue Mass

The results are summarised in Figure 3.5.7. Interscapular brown adipose tissue changes were believed to reflect changes at other brown adipose sites. The BAT was excised from the animals on day 42 of cold exposure.

**Figure 3.5.7** The Effects of Cold Acclimation With and Without Concomitant Hyperphagia on Interscapular BAT Mass

Values are mean±SEM for groups of 7 (cold ad libitum fed), 5 (cold pair fed) and 6 (warm control) rats. Symbol (**) indicate values significantly different from controls, **=p<0.01.

One-way analysis of variance of BAT mass followed by Scheffe's range test revealed that both cold acclimated groups of rats had significantly greater BAT masses than the warm control group. There was no significant difference in BAT mass between ad libitum fed and food restricted cold acclimated animals after 6 weeks cold exposure. Both cold treated groups had doubled their mass of interscapular BAT.
Hyperphagia of 36 days duration had no significant effect on intestinal wet weight in the cold *ad libitum* fed rats (Figure 3.5.8).

**Figure 3.5.8** The Effects of Cold Acclimation With and Without Concomitant Hyperphagia on Small Intestinal Wet Weight

Values are expressed as mean±SEM for groups of 7 (cold *ad libitum* fed), 5 (cold pair fed) and 6 (warm control) rats.

Relative intestinal weights were similar in the 3 study groups after 42 days of cold acclimation. The concentrations of IR-GIP and IR-GLP-1(7-36)amide have been calculated on a g wet weight/kg body weight basis to take into consideration the difference in size of the animals.
Figure 3.5.9 The Effects of Cold Acclimation With and Without Concomitant Hyperphagia on Small Intestinal IR-GIP

Values are expressed as mean±SEM for groups of 7 (cold *ad libitum* fed), 5 (cold pair fed) and 6 (warm control) rats. Symbol (0,00) indicates a value significantly different from the warm control group when assessed by one-way analysis of variance and Scheffe range test, 0=p<0.05; 00=p<0.01.

**Total Extractable IR-GIP**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IR-GIP (pmol/intestine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Rats</td>
<td>2000</td>
</tr>
<tr>
<td>Cold Ad Libitum Fed Rats</td>
<td>1500</td>
</tr>
<tr>
<td>Cold Pair Fed Rats</td>
<td>1000</td>
</tr>
</tbody>
</table>

**Intestinal IR-GIP Concentration**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IR-GIP (pmol/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Rats</td>
<td>100</td>
</tr>
<tr>
<td>Cold Ad Libitum Fed Rats</td>
<td>80</td>
</tr>
<tr>
<td>Cold Pair Fed Rats</td>
<td>50</td>
</tr>
</tbody>
</table>

Six weeks of cold acclimation with unrestricted food intake had no significant effect on the total amount of extractable IR-GIP but did reduce the relative concentration of IR-GIP in the small intestine (p<0.01). Restricted feeding and cold exposure also significantly reduced the concentration of IR-GIP in the small intestine of the pair-fed
rats (p<0.01). There was no significant difference between either of the cold-treated groups.

**Figure 3.5.10** The Effects of Cold Acclimation With and Without Concomitant Hyperphagia on Small Intestinal IR-GLP-1(7-36)amide

Values are expressed as mean±SEM for groups of 7 (cold *ad libitum* fed), 5 (cold pair fed) and 6 (warm control) rats.

**Total Intestinal IR-GLP-1(7-36)amide**

- **Control Rats**
- **Cold Ad Libitum Fed Rats**
- **Cold Pair Fed Rats**

Cold acclimation in the presence of hyperphagia had no significant influence on total extractable IR-GLP-1(7-36)amide or the relative concentration of IR-GLP-1(7-
3.5.4 Discussion

Over the first 6 days of cold exposure both the pair-fed and the *ad libitum* fed rats exhibited an initial stress reaction to the change in environmental temperature from 22°C to 4°C. The growth curve of both these groups of animals levelled off. The cold *ad libitum* fed rats responded with a rapid (49%) increase in food intake above normal. This subsequently increased the slope of their growth curve to mean values below, but statistically indistinguishable from, age-matched, warm acclimated control rats. The cold-exposed pair-fed rats, unable to increase food intake, exhibited retarded growth as their food consumption failed to meet the increased metabolic demand induced by cold exposure. Surprisingly, body weight changes never became negative unlike the weight loss observed with pair feeding in previous studies (Johnson *et al.*, 1982; Kuroshima & Yahata, 1985; Puerta & Abelenda, 1987). This may be attributable to differences in the dietary mixture utilised in the various studies and the relatively high food intake recorded by the warm control animals during this study.

Despite the differences in food intake between the two cold acclimated groups of rats, their relative interscapular BAT mass after 6 weeks cold exposure was comparatively similar. Both groups showed a mean doubling of the control values. This is in agreement with the findings of Kurishima & Yahata (1985) and Puerta & Abelenda (1987). The data supports the hypothesis that the improved cold tolerance upon prolonged cold exposure is related to the adaptation to the lower temperature rather than to an increased food intake.

Basal plasma measurements revealed that cold acclimation produced an increased plasma glucose concentration that developed faster in the presence of, but was not dependent on, hyperphagia. The increase in glucose was apparent 2-3 weeks
after cold exposure. Raised basal plasma glucose concentrations have also been reported by Cunningham et al. (1985). However other studies have reported no change from control values (Depocas & Masironi, 1960; Kuroshima et al., 1978) whilst a few have shown a fall in basal plasma glucose with cold acclimation (Beck et al., 1967; Harada et al., 1982).

Cold stress causing hypothermia can directly influence insulin release. Several mechanisms for its action have been proposed, including reduced potassium conductance by the B cell (Dawson et al., 1986; Atwater et al., 1984) or impaired calcium influx (Escolar et al., 1987). In the present study, 3 days of continuous cold exposure significantly depressed basal insulin levels in both cold treated groups, suggesting an acute cold stress effect. In the pair-fed cold exposed animals, plasma insulin levels remained markedly suppressed throughout the study period. This is most probably the result of the semi-starved state of these animals.

The cold hyperphagic animals, on the other hand, recovered normal basal plasma insulin levels around the same time as the hyperphagia became apparent. Fasting prior to the glucose challenge significantly lowered the insulin levels of these animals, suggesting that their insulin levels were normally dependent on the presence of hyperphagia. In warm acclimated animals, hyperphagia due to cafeteria feeding produces a hyperinsulinaemia which has been shown to be reversed by a cold environment (Vallerand et al., 1986). This effect was attributed to activation of the sympathetic innervation of the pancreas during cold exposure inhibiting insulin secretion. In contrast to the normal circulating basal insulin levels described in the present study, other workers have reported a progressive fall in pancreatic insulin output during cold acclimation in the presence of hyperphagia (Edwards & Howland, 1986). In the glucose challenge test, however, there was a progressive fall in the insulin response, supporting the idea of changes in insulin secretory patterns to be due to cold acclimation rather than exposure. The increased insulin sensitivity reported in
the cold hyperphagic animals is well documented and believed to be due to increased blood flow to the tissues controlled by sympathetic stimulation (Foster & Frydmann, 1979; Vallerand et al., 1986).

In comparison to the food-restricted, cold-acclimated animals, basal plasma IR-GIP concentrations in the cold-treated hyperphagic animals showed major changes over the 6-week period of cold acclimation. IR-GIP concentrations in the presence of cold exposure and hyperphagia achieved maximum concentrations on day 17 of the study (10 days after cold exposure began and 4 days after food intake significantly increased). These raised basal levels were maintained over the next 10 days and then subsequently fell, despite maintenance of the hyperphagia, to concentrations intermediate between the warm control and pair-fed animals. The cold-exposed pair-fed group had consistently low plasma IR-GIP levels reflecting their semi-starved state.

The cold hyperphagic animals demonstrated a classical adaptation response of an initial alarm reaction followed by a stage of resistance and finally reaching a stage of adaptation (Selye, 1976). In these animals it is possible that the rapid development of the hyperphagic condition caused an initial hyperstimulation of the K cell before adaptation or exhaustion of the K cell occurred. Measurements of intestinal entero-endocrine function in fully acclimated rats revealed that significant differences were present between cold acclimated and warm acclimated animals with regard to both IR-GIP and IR-GLP-1(7-36)amide. Hyperphagia, of 38 days' duration, in the cold ad libitum fed animals did not modify intestinal IR-GIP or IR-GLP-1(7-36)amide beyond the differences imposed by the cold treatment. Intestinal measurement revealed that the fall in plasma IR-GIP of the cold hyperphagic animals was not due to exhaustion of the K cell, but to some adaptive response.

Both GIP and GLP-1(7-36)amide are stimulated by a mixed meal (Kreymann et al., 1987). The observation that both these hormones are increased in the intestines of cold acclimated animals to a similar degree in spite of the differences in food
consumption would suggest that alternative or additional factors are mediating their increase. Insulin has been proposed to operate a negative feedback on GIP secretion (Brown et al., 1975). It may be speculated that a similar feedback mechanism could exist on GLP-1(7-36)amide secretion. Cold-acclimated, pair-fed animals had markedly reduced plasma insulin levels and the hyperphagic animals had a suppressed insulin response to a glucose challenge after prolonged cold exposure. Potentially the increase in intestinal IR-GIP and IR-GLP-1(7-36)amide could be due to modification of this feedback loop.

Cold acclimation takes three weeks before the animal is completely acclimated (Smith & Horwitz, 1969). Insulin secretion in cold adapted animals is strongly opposed by sympathetic stimulation and plasma glucagon has been reported to increase significantly as a result. Glucagon has potentially been implicated in direct BAT activation (Howland, 1986) but glucagon also strongly stimulates lipolysis in white adipose tissue, providing the metabolic fuel necessary for BAT activity. However, in the presence of a reduced insulin concentration lipolysis could well proceed at a faster rate than necessary or desirable. GLP-1(7-36)amide has been shown to inhibit glucagon release (Kreymann et al., 1987) and GIP has been shown to competitively block glucagon stimulated lipolysis (Ebert & Brown, 1976). Therefore, it could be speculated that these two hormones may be important in slowing the rate of lipolysis in the initial stages of cold exposure before BAT is capable of handling the increase in free fatty acids. In addition, GIP may also be involved in stimulating the increased activity of lipoprotein lipase (LPL) of white adipose tissue that has been reported in prolonged cold exposure (Deshaines et al., 1986). Increasing LPL activity in white adipose tissue would provide an additional source of free fatty acids.
GLP-1(7-36)amide also has the ability to stimulate fatty acid synthesis (Oben et al., 1991c). The potential roles of these hormones in cold acclimation has yet to be elucidated but their direct biological actions on adipose tissue may well be of equal if not greater importance with their incretin actions under these circumstances.
Conclusions

All four studies have looked at entero-endocrine function under different physiological situations for rats.

The pregnancy and lactation study revealed that the hyperphagia associated with these states was not the only influence on IR-GIP secretion as the acute IR-GIP response to nutrients was not grossly modified. The intestinal concentration of IR-GIP and IR-GLP-1(7-36)amide was significantly influenced by pregnancy, when the increase in food intake was only moderately increased. The insulin resistance of pregnancy may possibly be influencing entero-endocrine function. Down-regulation of the negative feedback removing a controlling influence on the K and possibly the L cell. Lactation has been reported to be an insulin-sensitive situation and an increased insulin feedback on the K and L cell may be responsible for off-setting the increased stimulation of the large rise in food intake. An increased GIP and GLP-1(7-36)amide response during lactation would not be desirable as it could reduce the supply of nutrients for milk synthesis. The possibility of GIP and GLP-1(7-36)amide being taken up into rat milk and its possible importance in the neonatal intestine remains to be established.

The ontogeny and litter size studies both indicated that the biological action of IR-GIP in white adipose tissue may well be of more importance than its incretin function during suckling. Rapid adipose deposition is essential for the rat in order to conserve heat loss and to provide an energy store. The importance of GLP-1(7-36)amide in this function has been suggested by studies in pigs. Plasma assays for GLP-1(7-36)amide are as yet not sensitive enough for use in rats.

The cold acclimation study showed an adaptive response of IR-GIP to cold acclimation. Again this study supported the assumption that the other biological actions
of GIP are of importance. To date no studies have been reported investigating any possible direct actions of GIP or GLP-1(7-36)amide on brown adipose tissue. This is an area of research requiring further study.

BAT is of course very important in neonatal life, particularly in rats. The increased IR-GIP response to acute challenge at this time would support the idea of a role for GIP and GLP-1(7-36)amide in the activation/proliferation of BAT. Alternatively both hormones may have a supporting role providing, through their action on white adipose tissue, the necessary fuel supply for the BAT. BAT has been reported to shut down during lactation in mice (Trayhurn et al., 1982). The same may well be true for rats. This may explain the absence of a modified GIP response to the increased food intake at this time.

A great deal of further work is necessary in this area. The incretin role of these two hormones has received most attention so far but other functions may well be of equal importance in physiological states.
Chapter 4.0

MEASUREMENT OF BASAL AND NUTRIENT STIMULATED PLASMA IR-GIP AND INTESTINAL ENTERO-ENDOCRINE FUNCTION IN PATHOPHYSIOLOGICAL STATES ASSOCIATED WITH HYPERPHAGIA
The following studies all demonstrate hyperphagic conditions where the normal insulin homeostasis pattern is grossly disturbed either by artificial (chemical or tumour induced) or spontaneous (autoimmune) means. The aims of the three studies were to investigate whether normal entero-endocrine K and L cell secretory functions were modified by the pathological conditions imposed on the animals. Each study will be considered separately and overall conclusions will be discussed at the end of the Chapter.

4.1 The Effects of an Insulinoma upon Basal and Nutrient Stimulated Plasma IR-GIP and Intestinal IR-GIP and IR-GLP-1(7-36)amide

4.1.1 Introduction

The NEDH rat insulinoma has been largely exploited as a model for investigations into insulin secretion and biosynthesis (Flatt & Swanston-Flatt, 1985). Subscapular transplantation of insulinoma fragments results in a highly vascularised, encapsulated tumour. Tumour development is associated with progressive hypoglycaemia, hyperinsulinaemia and hyperphagia (Flatt et al., 1987). The inability of the animal to maintain normoglycaemia eventually culminates in neuroglycopenia.

The aims of this study were to investigate how hyperphagia, hypoglycaemia and hyperinsulinaemia might influence the normal functioning of the GIP-secreting K cell in both the basal state and in response to acute nutrient stimulation. Small intestinal concentrations of IR-GIP and IR-GLP-1(7-36)amide were also measured in tumour-bearing and control NEDH rats for comparative purposes.
4.1.2 Methods

4.1.2.1 Animals and their Treatment

Thirty six (24 tumour bearing and 12 control) male, inbred, albino, New England Deaconess Hospital (NEDH) rats from the colony maintained at the University of Surrey Experimental Unit, were used at 8-12 weeks of age (approximately 275-350g body weight). Animals were caged in groups of 6 under conditions described in Section 2.1. Plasma measurements were performed in two studies. This was necessitated by the short time between transplantation and development of hypoglycaemia in insulinoma-bearing animals (less than three weeks following transplantation). It also ensured that the tests were performed on animals with similar sized tumours and therefore similar insulin levels. The first study investigated the effects of hyperphagia, hypoglycaemia and hyperinsulinaemia on GIP secretion in the acute and basal state. The second study looked at the effect of exogenous porcine GIP on insulin secretion.

4.1.2.2 Insulinoma Transplantation

The time course of transplantation is outlined in Figure 4.1.1. Cryogenically preserved insulinoma tissue was thawed and transplanted into recipient rats on two occasions before use to ensure an active insulin-producing tumour for the experiment. Tumours were transplanted by excision of the tumour from the cadaver donor rat, removal of the capsule to expose the tumour and then fine mincing of the tumour, with a scalpel, until it could be taken up into a 1ml syringe. Recipient rats were anaesthetised with ether and 0.1-0.15ml of minced tumour was implanted subcutaneously into the subscapular region using a 16 gauge needle.
During the first study, the effects of insulinoma development on food intake and body weight were monitored daily. In addition to the standard pellet diet, all insulinoma-bearing rats received D-glucose, given in their drinking water at 10% (w/v). This was included from day 13 post-transplantation onwards. The aim of this was to
provide an easily assimilated source of carbohydrate to offset the developing hypoglycaemia. The survival times of the rats were recorded and the weights of the tumours (including capsule) noted at autopsy.

Basal levels of glucose, IR-insulin and IR-GIP were measured in 6 insulinoma-bearing and 6 control rats on days 5, 8, 10 and 12 post-transplantation. Blood was sampled, between 0900-1200 hours, from the cut tail tip of non-fasted, conscious rats. Collection of blood and its handling has been discussed in Section 2.4.1.

The exact nature of the provocative tests was detailed in Section 2.2. In the first study, an oral fat challenge (51.2kJ/kg body weight) and an oral glucose challenge (51.2kJ/kg body weight) were administered to two groups of insulinoma-bearing rats and their controls on day 14 post-transplantation. Plasma was aliquoted for the determination of glucose, IR-insulin and IR-GIP.

In the second study, intraperitoneal glucose challenges were performed on day 14 post-transplantation. Intraperitoneal glucose (51.2kJ/kg body weight) with and without intraperitoneal porcine GIP (50μg/kg body weight) was administered simultaneously in the same volume used for the oral glucose challenge.

Because of the severity of the hyperinsulinaemia and corresponding hypoglycaemia of the insulinoma-bearing rats, all provocative tests were performed in the basal, non-fasted, state.

On day 17 post-transplantation in study one, insulinoma-bearing rats were fasted and blood samples collected at 30-minute intervals for up to two hours to illustrate the effect of food restriction on these animals.
Small intestines were obtained from insulinoma-bearing and control rats of the second study on day 16 post-transplantation. The process of excision and subsequent treatment has been detailed fully in Section 2.4.2.

4.1.3 Results

The first casualties in the insulinoma-bearing rats were recorded on day 13 of both studies. Mean survival of the rats in study one was 16 ± 3 days, (mean±SD for n=16).

4.1.3.1 Body Weight and Food Intake

Body weight was recorded throughout the study. The data are presented up until the start of the provocative tests, (day 14).

Figure 4.1.2 The Effects of an Insulin Secreting Tumour in NEDH Rats on Body Weight

Values are expressed as mean±SEM for groups of 6 rats. Tumour fragments (0.1ml/rat) were implanted subcutaneously in the subscapular region on day 0.
Repeated measures analysis of variance revealed that no significant differences in body weight were apparent between the insulinoma-bearing and control rats during the study period. Within each group over the study period body weight increased steadily but not significantly.

**Figure 4.1.3** The Effects of an Insulin Secreting Tumour on Food Intake in NEDH Rats

Values are expressed as mean±SEM for groups of 6 rats. Tumour fragments (0.1ml/rat) were implanted subcutaneously in the subscapular region on day 0. Symbol (**) indicate values significantly different from the control group, at that and subsequent time points, when assessed by repeated measures analysis of variance and Student's unpaired t-test, **=p<0.01.

Food consumption data showed significant differences between control and insulinoma-bearing animals over the study period, when assessed by repeated measures analysis of variance (p<0.01). Hyperphagia became established in the insulinoma-bearing animals at 10 days post-transplantation. Food consumption in the insulinoma rats continued to increase until day 13-14 post-transplantation. Between days 14-17, mean food intake was maintained at 45-50g/rat/24 hours in the insulinoma-bearing group.
4.1.3.2 Basal Levels of Plasma Glucose, IR-Insulin and IR-GIP in Insulinoma Bearing NEDH Rats

The regular sampling and assay of blood revealed the metabolic consequences of tumour development. The establishment of the tumour with its concomitant hyperphagia resulted in an increase in circulating plasma IR-insulin and plasma IR-GIP concentrations, with a precipitous fall in plasma glucose (Figure 4.1.4).

Repeated measures analysis of variance revealed that the changes in all three plasma variables over time were significant (glucose, \( p<0.01 \); IR-insulin, \( p<0.01 \); IR-GIP, \( p<0.01 \)). Student's unpaired t-test revealed that by 8 days post-transplantation the insulinoma-bearing animals had significantly elevated plasma IR-insulin levels. The depression in plasma glucose levels became significant two days later on day 10. Glucose concentrations continued to fall as plasma IR-insulin levels increased. Plasma IR-GIP levels were significantly elevated in the insulinoma rats on day 10 post-transplantation, the same day that the food intake of these animals became significantly increased. By the start of the provocative tests, the insulinoma-bearing rats exhibited marked hypoglycaemia, severe hyperinsulinaemia and hyperphagia.
Figure 4.1.4 The Effects of an Insulin Secreting Tumour on Plasma Glucose, Plasma IR-Insulin and Plasma IR-GIP in NEDH Rats

Values are expressed as mean±SEM for groups of 6 animals. Symbols (*,**) indicate values significantly different from the control group when assessed by Student's unpaired t-test, *=p<0.05; **=p<0.01.
4.1.3.3 Acute Nutrient Stimulation Tests

4.1.3.3.1 Oral Glucose Tolerance Test

The responses of the two groups to this challenge are summarised graphically in Figure 4.1.5.

As a result of not fasting the rats, the individual responses of the animals were very variable and the plasma values obtained at the zero time varied considerably between the insulinoma-bearing and the control rats. At all time points, the insulinoma-bearing animals had significantly lower plasma glucose levels and significantly higher plasma IR-insulin levels when compared to their controls. The incremental and integrated responses of the animals were calculated to aid comparison. These responses were calculated as described in Section 2.9. The results are summarised in Tables 4.1.1 and 4.1.2.
Figure 4.1.5 The Effects of the Oral Administration of Glucose (51.2kJ/kg body weight) in Insulinoma Bearing and Control NEDH Rats

Values are expressed as mean±SEM for groups of 6 animals. This test was administered 14 days post transplantation Symbol (**) indicates values significantly different from control values at that time point when assessed by Student's unpaired t-test, **=p<0.01.
### Table 4.1.1 Incremental Responses of Insulinoma Bearing and Control NEDH Rats to an Oral Glucose Challenge (51.2kJ/kg body weight)

Values are mean±SEM for groups of 6 rats. Incremental responses were calculated as described in Section 2.9. Symbols (*, **) indicate values significantly different from control values when assessed by means of Student's unpaired t-test, *=p<0.05; **=p<0.01.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Incremental Plasma Glucose Response (0-30 minutes) mmol/l</th>
<th>Incremental Plasma IR-Insulin Response (0-30 minutes) ng/ml</th>
<th>Incremental Plasma IR-GIP Response (0-30 minutes) pmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulinoma Bearing NEDH Rats</td>
<td>2.5 ± 0.4 *</td>
<td>113.3 ± 20.8 **</td>
<td>182.5 ± 29.7</td>
</tr>
<tr>
<td>Control NEDH Rats</td>
<td>4.9 ± 0.7</td>
<td>5.2 ± 0.2</td>
<td>118.3 ± 29.7</td>
</tr>
</tbody>
</table>

### Table 4.1.2 Integrated Responses of Insulinoma Bearing Rats to an Oral Glucose Challenge (51.2kJ/kg body weight)

Values are mean±SEM for groups of 6 rats. Integrated responses were calculated as described in Section 2.9. Symbol (**) indicates values significantly different from control values when assessed by means of Student's unpaired t-test, **=p<0.01.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Integrated Plasma Glucose Response (0-120 minutes) (mmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-Insulin Response (0-120 minutes) (ng/ml).hr⁻¹</th>
<th>Integrated Plasma IR-GIP Response (0-120 minutes) (pmol/l).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulinoma Bearing NEDH Rats</td>
<td>17.9 ± 21.6</td>
<td>2420.5 ± 873.9 **</td>
<td>2200 ± 1600</td>
</tr>
<tr>
<td>Control NEDH Rats</td>
<td>139.8 ± 30.8</td>
<td>125.5 ± 13.1</td>
<td>4100 ± 900</td>
</tr>
</tbody>
</table>

The insulinoma-bearing animals were able to respond to the oral glucose challenge with a significantly greater insulin response than the control rats. As a
consequence of their excessive insulin concentrations the incremental plasma glucose response was significantly attenuated. When the marked difference in basal GIP levels is excluded there was no difference in the GIP response of the insulinoma-bearing and control rats either incrementally or expressed as the integrated response.

4.1.3.3.2 Oral Fat Tolerance Test

The results of this test are summarised in Figure 4.1.6.

Oral fat administration did not significantly modify plasma insulin concentrations in either the insulinoma-bearing or the control groups. Plasma glucose fell significantly, over the time course of the test, in the insulinoma animals (within group repeated measures analysis of variance p<0.01). Integrated responses have been calculated to aid comparison of the two groups; these are summarised in Table 4.1.3.
The Effects of the Oral Administration of a Corn Oil Suspension (51.2kJ/kg body weight) on Insulinoma Bearing and Control NEDH Rats

Values are expressed as mean±SEM for groups of 6 animals. This test was performed 14 days post transplantation. Symbol (**) indicates values significantly different from control values at that time point when assessed by Student's unpaired t-test, **=p<0.01.

- Control NEDH Rats
- Insulinoma Bearing NEDH Rats
Table 4.1.3 Integrated Responses of Insulinoma Bearing and Control NEDH Rats to an Oral Fat Challenge (51.2kJ/kg body weight)

Values are mean±SEM for groups of 6 rats. Integrated responses were calculated as detailed in Section 2.9. Symbol (*) indicate values significantly different from control values when assessed by means of Student's unpaired t-test, *=p<0.05.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Integrated Plasma Glucose Response (0-120 minutes) (mmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-Insulin Response (0-120 minutes) (ng/ml).hr⁻¹</th>
<th>Integrated Plasma IR-GIP Response (0-120 minutes) (pmol/l).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulinoma Bearing NEDH Rats</td>
<td>-31.4 ± 8.9 *</td>
<td>-906.2 ± 649.3</td>
<td>11200 ± 2300 *</td>
</tr>
<tr>
<td>Control NEDH Rats</td>
<td>8.4 ± 7.9</td>
<td>20.1 ± 23.6</td>
<td>3200 ± 1200</td>
</tr>
</tbody>
</table>

The integrated insulin responses of the insulinoma-bearing animals were very variable. As a result the standard error of the response was very great and masked significant differences. An oral fat challenge significantly reduced the plasma glucose concentration of the tumour-bearing rats; the excessive insulin levels also fell during the test but were still extremely high. The acute plasma IR-GIP response of the insulinoma-bearing animals was heightened in comparison with the control rats.

4.1.3.3.3 Intraperitoneal Glucose Challenge Tests

These tests were performed in study two. The basal IR-insulin levels of the insulinoma-bearing animals in this study were more variable than the levels seen in study one. The test was performed in the presence and absence of porcine GIP. Plasma was collected for glucose and IR-insulin assay only.
Figure 4.1.7  The Effects of an Insulin Secreting Tumour on the Plasma Glucose Response to an Intraperitoneal Glucose and GIP Challenge (51.2kJ and 50μl/kg body weight respectively) in NEDH Rats

Values are mean±SEM for groups of 5 rats. Symbols (●, ●) indicate values significantly different from control values when assessed by means of Student's unpaired t-test, ●=p<0.05; ●●=p<0.01.

Control NEDH Rats

Insulinoma Bearing NEDH Rats

For both the control and insulinoma-bearing rats glucose tolerance was improved in the presence of porcine GIP, significantly so for the insulinoma-bearing
animals (repeated measures analysis of variance p<0.01). This is confirmed by the
significant difference in the integrated glucose responses for the insulinoma-bearing
animals (Table 4.1.4).

Figure 4.1.8 The Effects of an Insulin Secreting Tumour on the Plasma IR-Insulin Response to an Intraperitoneal Glucose and GIP Challenge (51.2kJ and 50μg/kg body weight respectively) in NEDH Rats
Values are mean±SEM for groups of 5 rats. Symbols (*, **) indicate values significantly different from control values when assessed by means of Student's unpaired t-test, *=p<0.05; **=p<0.01.

Control NEDH Rats

![Graph of Plasma IR-Insulin response for Control NEDH Rats]

Insulinoma Bearing NEDH Rats

![Graph of Plasma IR-Insulin response for Insulinoma Bearing NEDH Rats]
The improvement in glucose tolerance, for both groups, in the presence of GIP, was achieved by an increased peak insulin secretion. However the large individual variation in the insulinoma-bearing animals' response masks any significant difference in the integrated response of these animals (Table 4.1.4). The control rats had a significantly greater total IR-insulin response in the presence of porcine GIP.

**Table 4.1.4** Integrated Plasma Glucose and IR-Insulin Responses of Insulinoma Bearing Rats and Their Controls

Values are mean±SEM for groups of 5 rats. Integrated responses were calculated as described in Section 2.9. Symbol (*) indicates values significantly different from response to IP Glucose alone when assessed by means of Student's unpaired t-test, *=p<0.05.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>IP Glucose Response (0-120 minutes) (mmol/l).hr⁻¹</th>
<th>IP Plasma IR-Insulin Response (0-120 minutes) (ng/ml).hr⁻¹</th>
<th>IP Plasma Glucose Response (0-120 minutes) (mmol/l).hr⁻¹</th>
<th>IP Plasma IR-Insulin Response (0-120 minutes) (ng/ml).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulinoma Bearing NEDH Rats</td>
<td>465.5 ± 49.2</td>
<td>3212.4 ± 1311</td>
<td>213.8 ± 33.2</td>
<td>1562.9 ± 2263.5</td>
</tr>
<tr>
<td>Control NEDH Rats</td>
<td>806.5 ± 102.9</td>
<td>89.3 ± 19.8</td>
<td>489.5 ± 71.7</td>
<td>170.6 ± 22.6</td>
</tr>
</tbody>
</table>

4.1.3.4 The Effects of Food Withdrawal on Basal Levels of Plasma Glucose, IR-Insulin and IR-GIP in Insulinoma Bearing NEDH Rats

This was performed on day 17 post-transplantation. The results are summarised in Figure 4.1.9.
Figure 4.1.9  The Effects of the Removal of the Food Supply on Insulinoma Bearing NEDH Rats

Values are expressed as mean±SEM for groups of 5 rats. Symbols (*, **) indicate values significantly different from time 0 as assessed by Student’s paired t-test, *=p<0.05; **=p<0.01.
On removal of the food supply from the insulinoma-bearing rats there was a rapid and significant fall in plasma glucose. This decline in plasma glucose continued throughout the two hours. Plasma IR-insulin concentrations declined on the removal of food, but the decrease in concentration was only significant after 90 minutes. The levels at the end of the two hour period were still exceptionally high. Plasma IR-GIP declined rapidly and significantly over the first hour of the experiment but after two hours the levels were still comparable with basal levels of the control NEDH rats.

4.1.3.5 The Effects of an Insulin Secreting Tumour on Small Intestinal Entero-Endocrine Function in NEDH Rats

Hyperphagia of six days' duration preceded the excision of the small intestines. The influence of the pathological condition of tumour development on intestinal wet weight is summarised in Figure 4.1.10.

Figure 4.1.10 The Effects of an Insulin Secreting Tumour on Small Intestinal Wet Weight in NEDH Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbol (**) indicates a value significantly different from the control group when assessed by Student's unpaired t-test, **=p<0.01.

- Control NEDH Rats
- Insulinoma Bearing NEDH Rats
Actual intestinal wet weight was increased 30% in the insulinoma-bearing animals. Relative intestinal weight was also significantly increased in the insulinoma-bearing animal after 6 days of hyperphagia. Body weights were similar between the two groups, insulinoma-bearing animals being slightly, but not significantly, heavier than the control animals.

Figure 4.1.11  
The Effects of an Insulin Secreting Tumour on Small Intestinal IR-GIP in NEDH Rats

Values are expressed as mean±SEM for groups of 6 rats.

**Total Extractable IR-GIP**

<table>
<thead>
<tr>
<th></th>
<th>Control NEDH Rats</th>
<th>Insulinoma Bearing NEDH Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal IR-GIP Concentration</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IR-GIP (pmol/ intestine)**

**IR-GIP (pmol/g wet wt/kg body wt)**
The growth of an insulinoma together with six days of hyperphagia had no significant effect either on total extractable GIP content of the small intestine or its actual concentration (pmol/g wet weight) or its relative concentration (pmol/g wet weight/kg body weight).

**Figure 4.1.12** The Effects of an Insulin Secreting Tumour on Small Intestinal IR-GLP-1(7-36)amide in NEDH Rats

Values are expressed as mean±SEM for groups of 6 rats.

**Total Extractable IR-GLP-1(7-36)amide**

- ■ Control NEDH Rats
- □ Insulinoma Bearing NEDH Rats

**Intestinal IR-GLP-1(7-36)amide Concentration**

- ■ Control NEDH Rats
- □ Insulinoma Bearing NEDH Rats
Insulinoma growth and hyperphagia had no significant influence on total extractable IR-GLP-1(7-36)amide or its actual or relative concentration in the small intestine.

4.1.4 Discussion

The absence of a significant increase in body weight despite hyperphagia and hyperinsulinaemia in the insulinoma animals reflects the strong influence of malignant disease. By day 13, the tumour itself could account for an average 1.7% of body weight (as estimated an autopsy): by day 17, this had risen to 2.4%. At autopsy the tumours were observed to be more invasive and not as well encapsulated as previous studies of tumour growth would indicate (Flatt et al., 1986). The maximum insulin levels previously reported in this colony of rats after insulinoma transplantation was 40ng/ml (Flatt et al., 1987). Much higher values are recorded here. These observations together with the fact that the first mortalities occurred on day 13, rather than 21-22 days post-transplantation as previously reported (Flatt et al., 1987) suggest the possibility that the growth characteristics of the tumour are gradually changing.

Sequential plasma measurements revealed the onset of hypoglycaemia and hyperinsulinaemia preceded the hyperphagia that was sustained throughout the rest of the study. The hyperphagia was insufficient to maintain normoglycaemia in these animals. A two-hour withdrawal of the food supply demonstrated that it was sufficient to defer precipitation of a hypoglycaemic coma. Mean plasma IR-GIP levels were higher in the insulinoma animals when compared to the control animals from day 10 post-transplantation onwards, when the hyperinsulinaemia was already established. Fasting demonstrated that regular food intake was necessary to maintain the high plasma IR-GIP levels. Changes in plasma IR-GIP were not reflected in intestinal IR-GIP measurements. It is therefore possible to suggest that the high basal IR-GIP
levels seen in the insulinoma-bearing animals are due to the increased, regular, food consumption seen in these animals.

The basic defect in insulinomas is the reduced capacity for insulin storage resulting in the exocytosis of immature secretory vesicles. There is therefore a marked increase in circulating inactive pro-insulin which unfortunately will cross-react in the assay system utilised here. In human insulinomas the amount of circulating pro-insulin can rise to up to 80% of circulating insulin levels (Clarke & Hales, 1991). The measured basal insulin levels of the insulinoma-bearing rats were excessively high. The fact that the animals survived for a period of time with these high levels would suggest that a significant proportion of the insulin was inactive pro-insulin and also that the animals built up a degree of insulin resistance. The tumour, though self regulating, was sensitive to external influences. Fasting revealed that food was contributing to the hyperinsulinaemia. Hyperphagia was therefore offsetting the hypoglycaemic effects of the insulinoma but also promoting this effect by stimulating insulin secretion.

Fasting was seen to produce a rapid deterioration in the metabolic state of the insulinoma-bearing animals. All provocative tests were necessarily performed in the fed state. This fact created experimental problems in the gavage administered tests as it is difficult to administer a bolus dose of a solution to an already full stomach. The other associated problem was the wide variation in responses to the test solutions seen in the animals. This is not simply a difficulty of the protocol of this experiment as the same problem has been reported by other workers in both animal and human studies (Go et al., 1979; Morgan, 1979).

In the acute provocative oral and intraperitoneal glucose tests, the insulinoma-bearing animals showed a persistent depression of plasma glucose. Maximal concentrations achieved corresponded to only modest hyperglycaemic values in spite of
the large dose of glucose administered. This is due to the rapid uptake of glucose into
the tissues stimulated by insulin.

The greatest stimulus for IR-GIP secretion in the control rats was oral fat and
the same was true for the insulinoma-bearing rats. The hyperinsulinaemia failed to
prevent fat stimulated GIP secretion in a hypoglycaemic environment. This finding
supports the results reported by Go et al. (1979) who similarly found in humans with
insulinomas that GIP secretion in response to oral fat was not suppressed. IR-GIP
secretion in response to fat stimulation failed to stimulate insulin secretion because of
the hypoglycaemic environment.

The oral glucose tolerance test produced a normal IR-GIP response and insulin
secretion was stimulated in the insulinoma-bearing animals. The intraperitoneal tests
revealed that the tumour could not significantly respond to glucose alone, a finding
supported by the results of Tan et al. (1986). The tumour was however sensitive to
GIP under the same experimental conditions. Other workers have also demonstrated
that insulinomas are sensitive to GIP and other secretagogues, namely glucagon-like
peptide-1 fragments, which utilise the same secondary messenger system (Tan et al.,
1986; Flatt et al., 1990). Specific GIP receptors have been reported in human
insulinoma plasma membrane (Maletti et al., 1987).

Intestinal measurements revealed that hyperphagia of 6 days' duration had
caused a significant increase in relative and actual small intestinal wet weight. A similar
finding was reported by Conlon and colleagues (1986). However, the insulinoma had
no effect on the GIP or GLP-1(7-36)amide content of the small intestine. In contrast,
the small intestinal content of enteroglucagon measured with N and C terminal specific
glucagon antisera has been reported to be increased in insulinoma-bearing rats 14 days'
after tumour transplantation (Conlon et al., 1986). The reason for this discrepancy may
be differences in the specificity of the assay systems employed.
4.2 The Effects of Streptozotocin Induced Insulin Deficiency on Basal and Nutrient Stimulated Plasma IR-GIP and Intestinal Entero-Endocrine Function in Wistar Rats

4.2.1 Introduction

Streptozotocin is utilised as an experimental diabetogenic agent, producing irreversible damage to pancreatic B cells. Following acute injection there is an initial rise in blood sugar in the first 4 hours, followed by hypoglycaemia between 6-12 hours and finally permanent hyperglycaemia 24 hours post treatment (Agarwal, 1980). Diabetes mellitus induced by high dose streptozotocin is comparable with insulin dependent diabetes mellitus (IDDM) in humans (Leslie, 1983). Streptozotocin is an acknowledged method for inducing experimental diabetes (Agarwal, 1980; Cooperstein & Watkins, 1981).

As reviewed in Section 1.6.2, there are studies indicating both increased and decreased IR-GIP concentrations in IDDM. Animal models of IDDM have not been exploited fully in this area of research (Kreymann et al., 1988). The aim of this investigation was to establish the function of an endocrine component of the EIA in hypoinsulinaemic, streptozotocin induced diabetes in rats.

4.2.1 Methods

4.2.1.1 Animals and their Treatment

Twenty male, Wistar albino rats from the colony maintained at the University of Surrey were utilised for this study at approximately 10 weeks of age (170-190g). The animals were caged in groups of 3 and housed as described in Section 2.1. Food and water were provided ad libitum as discussed previously, Section 2.1.
4.2.1.2 Experimental Procedures

Insulin deficiency was induced in two groups of fed animals (n=6) by intraperitoneal administration of a streptozotocin solution (Mixed Anomers N° S-0130, Sigma Chemical Company, Poole, England). The streptozotocin, at a dose of 65mg/kg body weight, was solubilised in 0.5M citrate buffer, pH 4.5, immediately prior to injection. Untreated age matched rats were used as controls.

Body weight and food intake were monitored daily. Sequential blood samples (200µl) were regularly taken at 0900-1200 hours from the tail tip of non-fasted conscious rats for plasma glucose, IR-insulin and IR-GIP analysis. At 16 days post streptozotocin administration, the rats were fasted for 18 hours and then subjected to an oral glucose tolerance test (51.2kJ/kg body weight) or an oral fat tolerance test (51.2kJ/kg body weight). The exact nature of these tests was described in Section 2.2.

On day 24, the rats were fasted for 18 hours and glucose (51.2kJ/kg body weight) or glucose with simultaneous porcine GIP (51.2kJ/kg body weight and 50µg/kg body weight respectively) were administered by intraperitoneal injection. Blood samples were collected for glucose and IR-insulin assay.

On day 28, the rats were fasted for 18 hours and the oral glucose and oral fat tolerance tests were repeated in the presence of exogenous porcine insulin [(2U/ml) per kg body weight of monocomponent porcine insulin, Actrapid, Novo Industria, Copenhagen, Denmark]. On day 32, the rats were fasted for 18 hours prior to administration of porcine insulin either alone, at the same dose as previously used, or with porcine GIP (50µg/kg body weight).
Finally, 38 days after the streptozotocin administration, both the insulin deficient and control rats were killed by cervical dislocation and the whole of their small intestines excised and treated as described in Section 2.4.2.

4.2.3 Results

The streptozotocin-treated rats (STZ) were tail bled 3 days after streptozotocin administration and the small sample of whole blood was immediately tested using a glucose reagent impregnated strip (Visidex Reagent Strips, Ames Division, Miles Laboratories Ltd., Slough). The streptozotocin-treated rats all gave blood glucose results in the range 6.7-11 mmol/l, indicating that hyperglycaemia was established. In comparison the control rats gave blood glucose values in the range (2.2-3.9 mmol/l). It must be noted that the reagent strips were old and it was the difference between the streptozotocin-treated and control groups that indicated that the animals were diabetic rather than the absolute glucose values.

4.2.3.1 Body Weight and Food Intake

The body weight results are shown up to the start of the fasting and provocative tests on day 15. Differences between the two groups at the start of the provocative tests were maintained until the study was concluded. The results are summarised graphically in Figure 4.2.1.
The Effects of a Single Large Dose of Streptozotocin (65mg/kg body weight) on Body Weight in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Streptozotocin was administered on day 0. Symbols (*, **) indicate significant differences between the two groups when assessed by Student's unpaired t-test, *=p<0.05; **=p<0.01.

The streptozotocin-treated rats failed to gain weight at the same rate as the control rats. Repeated measures analysis of variance revealed differences both between and within the two groups over time were significant (p<0.01). Within the streptozotocin-treated group there was a wide variation in individual body weight gain, some animals maintaining their body weight whilst others lost weight over the study period.
The Effects of a Single Large Dose of Streptozotocin (65mg/kg body weight) on Food Intake in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Streptozotocin was administered on day 0. Symbol (**) indicates values significantly different from the control group at that time point when assessed by Student's unpaired t-test, **=p<0.01.

Repeated measures analysis of variance revealed that the differences in food intake that developed between the two groups were significant (p<0.01). The streptozotocin-treated rats were hyperphagic by day 7 and this hyperphagia persisted until the experiment was terminated, although food intake in both groups was disturbed by fasting and the acute test procedures.

Fluid intake was not recorded in the study but showed a remarkable increase over the first four days after streptozotocin administration and a high level of intake was present throughout the study.
4.2.3.2 Basal Levels of Plasma Glucose, IR-Insulin and IR-GIP in Streptozotocin Treated Wistar Rats

The regular sampling and assay of blood revealed the metabolic consequences of irreversible damage to the B cells. Repeated measures analysis of variance revealed significant changes in plasma glucose, IR-insulin and IR-GIP in the streptozotocin-treated group over time (p<0.01).

Three days after streptozotocin administration plasma IR-insulin levels were severely suppressed and correspondingly plasma glucose levels grossly elevated in these animals. A significant rise in basal plasma IR-GIP concentrations was observed in all streptozotocin-treated animals 7 days after streptozotocin administration. The rise in plasma IR-GIP followed the development of the hypoinsulinaemia and hyperglycaemia and occurred at the same time that food intake became significantly increased in these animals.
The Effects of Acute Streptozotocin Administration on Basal Plasma Glucose, IR-Insulin and IR-GIP in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbols (*, **) indicate values significantly different from the control group when assessed by Student's unpaired t-test, *=p<0.05; **=p<0.01.
4.2.3.3 The Effects of Food Withdrawal on Basal Levels of Plasma Glucose, IR-Insulin and IR-GIP in Streptozotocin Treated Wistar Rats

Fasting of the streptozotocin-treated and control rats for 18 hours prior to the provocative tests helped to standardise the plasma glucose and IR-GIP levels at the start of these tests. The mean results of fasting are summarised in Figure 4.2.4.

An 18 hour fast lowered the plasma glucose and IR-GIP levels in the streptozotocin-treated animals back to levels similar to those of the control rats. Fasting also lowered plasma IR-insulin concentrations in both the control and the streptozotocin-treated groups, however significant differences between the two groups persisted after the 18 hour fast.
**Figure 4.2.4**
Comparison of Plasma Glucose, IR-Insulin and IR-GIP Concentration in Fed and Fasted Streptozotocin Treated Diabetic Rats and their Controls

Values are expressed as mean±SEM for 6 observations in fed rats and 12 observations in fasted rats. Symbol (**) indicates values significantly different from the other three values when assessed by one-way analysis of variance followed by Scheffe's range test, **=*p<0.01. Symbol ○ indicates a value significantly different from the fasted control value, ○=*p<0.05.

![Graphs showing plasma glucose (mmol/L), plasma IR-Insulin (ng/ml), and plasma IR-GIP (pmol/l) levels in control and STZ treated rats.](attachment:image.png)
4.2.3.4  **Acute Nutrient Stimulation Tests**

4.2.3.4.1  **Oral Glucose Tolerance Test**

The responses of the streptozotocin diabetic rats, acute insulin-treated streptozotocin diabetic rats and the control rats are summarised in Figure 4.2.5.

Glucose tolerance was severely impaired in the streptozotocin-treated diabetic rats. The plasma IR-insulin response of these animals was small and insufficient to curtail the rise in plasma glucose. The integrated insulin response of these streptozotocin-treated animals was significantly reduced in comparison with the insulin response of the control animals, Table 4.2.1.
Figure 4.2.5 The Effects of the Oral Administration Of Glucose (51.2kJ/kg body weight) on Streptozotocin Treated Wistar Rats with and without Acute Insulin Treatment (2U/kg body weight)

Values are expressed as mean±SEM for groups of 6 rats. Insulin was administered at time 0. Symbols (*, **) indicate values significantly different from the two other groups when assessed by one-way analysis of variance followed by Duncan’s range tests, * = p<0.05; ** = p<0.01. Plasma IR-Insulin concentrations were not measured in those animals treated with exogenous insulin, symbols (*, **) in this graph indicate values significantly different from control values when assessed by Student’s unpaired t-test, * = p<0.05; ** = p<0.01.
Table 4.2.1 **Integrated IR-Insulin Response of Streptozotocin Treated Rats to an Oral Glucose Challenge (51.2kJ/kg body weight)**

Values are mean±SEM for groups of 6 rats. Symbol (**) indicates a value significantly different from the control value when assessed by Student's unpaired t-test, **=p<0.01. Integrated response was calculated as described in Section 2.9.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Integrated Plasma IR-Insulin Response (0-120 minutes) (ng/ml).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptozotocin-Treated Rats</td>
<td>32.63 ± 8.66 **</td>
</tr>
<tr>
<td>Control Rats</td>
<td>117.96 ± 16.95</td>
</tr>
</tbody>
</table>

The plasma IR-GIP response was significantly elevated above the normal response in the streptozotocin-treated animals. Acute administration of insulin to streptozotocin-treated animals immediately prior to the glucose load was sufficient to normalise their glucose tolerance. Plasma glucose levels in these insulin-treated animals actually fell significantly below control values by the end of the experiment. Repeated measures analysis of variance revealed highly significant differences in the plasma IR-GIP response of the three study groups (p<0.01). Insulin-treated streptozotocin diabetic animals' plasma IR-GIP response was normalised at 30 minutes and significantly suppressed to below control values at 60 and 120 minutes.
4.2.3.4.2 Oral Fat Tolerance Test

The responses of the streptozotocin diabetic, acute insulin-treated streptozotocin diabetic and the control rats are summarised in Figure 4.2.6.

Oral administration of corn oil did not modify plasma glucose or plasma IR-insulin concentrations in either the streptozotocin-treated or the control groups. Plasma IR-GIP responses were also similar over the first 60 minutes of the experiment for both of these groups. The tendency towards an increased IR-GIP response in the streptozotocin-treated animals became significant at 120 minutes. Administration of insulin to the streptozotocin-treated rats immediately prior to this test caused a severe reduction in plasma glucose levels. The plasma IR-GIP response of insulin-treated animals, to oral fat, was normalised.
Figure 4.2.6 The Effects of the Oral Administration of Corn Oil Suspension (51.2kJ/kg body weight) on Streptozotocin Treated Wistar Rats with and without Acute Insulin Treatment (2U/kg body weight)

Values are expressed as mean±SEM for groups of 6 rats. Symbols (*, **) indicate values significantly different from the two other groups when assessed by one-way analysis of variance followed by Duncan's range tests, * = p<0.05; ** = p<0.01. Plasma IR-insulin concentrations were not measured in those animals treated with exogenous insulin, symbols (*, **) in this graph indicate values significantly different from control values when assessed by Student's unpaired t-test, * = p<0.05; ** = p<0.01.
4.2.3.4.3 **Intraperitoneal Glucose Challenge Test**

This challenge was performed in the presence and absence of porcine GIP. Plasma was collected for glucose and insulin assay only.

**Figure 4.2.7** The Effects of Acute Streptozotocin Treatment in Wistar Rats on the Plasma Glucose Response to an Intraperitoneal Glucose Challenge (51.2kJ/kg body weight) with and without Concomitant Porcine GIP (50μg/kg body weight)

Values are expressed as mean±SEM for groups of 6 rats. Symbol (**) indicates a significant difference when assessed by Student's unpaired t-test, **=p<0.01.

**Control Rats**

![Graph showing plasma glucose levels for control rats with and without GIP](image)

**Streptozotocin Diabetic Rats**

![Graph showing plasma glucose levels for diabetic rats with and without GIP](image)
The streptozotocin-treated animals showed an impaired glucose tolerance in both experiments when compared with the control animals' response. Comparison with the results from the oral glucose challenge revealed that even though the dose of glucose used was the same, very different responses were observed both in the test and control animals. Glucose tolerance was improved for both the streptozotocin-treated and the control rats in the presence of porcine GIP. The improvement was far greater in the control animals, as indicated by comparison of integrated glucose responses, Table 4.2.2.
Figure 4.2.8 The Effects of Acute Streptozotocin Treatment in Wistar Rats on the Plasma IR-Insulin Response to an Intraperitoneal Glucose Challenge (51.2kJ/kg body weight) with and without Concomitant Porcine GIP (50μg/kg body weight)

Values are expressed as mean±SEM for groups of 6 rats. Symbol (**) indicates a significant difference when assessed by Student's unpaired t-test, **=p<0.01.

Control Rats

Streptozotocin Diabetic Rats
Only the control rats showed an increased (28%) insulin response in the presence of exogenous GIP. The streptozotocin-treated rats were unable to increase their total insulin secretion above that seen with glucose alone.

**Table 4.2.2** Integrated Plasma Glucose and IR-Insulin Responses of Streptozotocin Treated Rats to an Intraperitoneal Glucose Challenge (51.2kJ/kg body weight) with and without Concomitant Porcine GIP Administration (50μg/kg body weight)

Values are mean±SEM for groups of 6 rats. Symbol (**) indicates a value significantly different from the control value when assessed by Student's unpaired t-test, **=p<0.01. Symbol (00) indicates a value significantly different from the response to glucose alone, 00=p<0.01.

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<thead>
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<th>Study Group</th>
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</tr>
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<tbody>
<tr>
<td></td>
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<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Response</td>
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<tr>
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<td>(0-120 minutes)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>33.7±15.1</td>
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<td>416±34.8</td>
</tr>
<tr>
<td></td>
<td>185.9±13.6</td>
<td>237.9±19.4</td>
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</table>
Figure 4.2.9 The Effects of Streptozotocin Treatment in Wistar Rats on the Plasma Glucose and IR-GIP Response to an Intraperitoneal Insulin Challenge [(2U/ml) per kg body weight] with and without Concomitant Porcine GIP (50µg/kg body weight)

Values are expressed as mean±SEM for groups of 6 rats. Plasma IR-GIP was not measured in the group administered exogenous GIP. Symbol (**) indicates a significant fall from the zero value in all 3 groups **=p<0.01.

Plasma Glucose

![Plasma Glucose Graph]

Plasma IR-GIP

![Plasma IR-GIP Graph]

Administration of insulin in the fasted state to streptozotocin-treated and control rats caused a fall in plasma glucose concentration in both groups over the first 30
minutes of the test \( (p<0.05) \). Plasma IR-GIP concentrations were not markedly affected by insulin in either the control or streptozotocin diabetic animals. When porcine GIP was administered with the insulin to streptozotocin diabetic rats there was no significant alteration in the plasma glucose disposal rate from that seen with insulin alone.

4.2.3.5 The Effects of Streptozotocin Induced Insulin Deficiency on Small Intestinal Entero-Endocrine Function in Wistar Rats

Hyperphagia of 31 days' duration preceded the excision of the animals' small intestines. The influence of streptozotocin damage to the B cell on intestinal wet weight is summarised in Figure 4.2.10.

**Figure 4.2.10** The Effects of Streptozotocin Induced Insulin Deficiency on Small Intestinal Wet Weight in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbols (**) indicate a value significantly different from the control value when assessed by Student's unpaired t-test, \( **=p<0.01 \).

![Intestinal Wet Weight Graph](image)

Actual intestinal weight was significantly increased in the streptozotocin-treated animals. Expressed on a body weight basis to take into account the difference in size of
the animals, relative intestinal wet weight was significantly increased in the streptozotocin-treated animals.

**Figure 4.2.11** The Effects of Streptozotocin Induced Insulin Deficiency on Small Intestinal IR-GIP in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbols (**) indicate a value significantly different from the control value when assessed by Student's unpaired t-test, **=p<0.01.

**Total Extractable IR-GIP**

**Intestinal IR-GIP Concentration**
Streptozotocin-induced insulin deficiency with its concomitant hyperphagia resulted in an increase in the small intestinal IR-GIP content and maintained the concentration of IR-GIP in spite of the large increase in intestinal weight.

**Figure 4.2.12** The Effects of Streptozotocin Induced Insulin Deficiency on Small Intestinal IR-GLP-1(7-36)amide in Wistar Rats

Values are expressed as mean±SEM for groups of 6 rats. Symbols (*,**) indicate a value significantly different from the control value when assessed by Student's unpaired t-test, *p<0.05; **p<0.01.

**Total Extractable IR-GLP-1(7-36)amide**

**Intestinal IR-GLP-1(7-36)amide Concentration**
Streptozotocin-induced insulin deficiency and its concomitant hyperphagia caused a significant increase in both the relative content and concentration of intestinal GLP-1(7-36)amide.

4.2.4 Discussion

Streptozotocin induces damage to the B cells which is irreversible. The rat is a particularly sensitive species to the effects of this compound. Streptozotocin was administered in the fed state. There is some evidence that glucose may influence the degree of damage to the B cell. The extent of insulin deficiency induced in the treated animals was variable even though the streptozotocin was administered on a body weight basis. This may possibly be due to the influence of the nutritional state of the animals. The resultant condition of the animals is due almost entirely to the affinity of streptozotocin for the B cell. The streptozotocin-treated rats exhibited hypoinsulinaemia, hyperglycaemia and impaired body weight gain in spite of the presence of hyperphagia. Polydipsia was also noted.

The hyperglycaemia of streptozotocin-induced diabetes is the consequence of the excess food consumption and insulin deficiency. Plasma IR-GIP levels were also elevated possibly as a result of the increased nutrient intake stimulating K cell secretion. The strong relationship between food intake and hyperglycaemia and enhanced K cell stimulation was revealed by an 18 hour fast. Fasting reduced the plasma glucose and IR-GIP levels back to control values.

GIP has been shown to stimulate glucagon release under basal conditions (Kreymann et al., 1987). This raised the question of whether GIP could contribute to the hyperglycaemia through elevation of glucagon concentrations. Administration of exogenous porcine GIP in the fasted state with intraperitoneal insulin failed to influence the plasma glucose levels of the streptozotocin-treated rats compared with the
effects of insulin alone. In the intraperitoneal glucose challenge, the observation that exogenous GIP failed to improve the insulin response of the streptozotocin-treated rats to an intraperitoneal glucose load illustrates the grossly compromised insulin secretory capacity of these animals.

The oral glucose tolerance test revealed a smaller intolerance to oral rather than intraperitoneal glucose. Addition of GIP to the intraperitoneal test revealed that differences in IR-GIP secretion could not account for the difference in glucose disposal rate, a finding that was also reported by Bailey and colleagues (1986b) in mice. The difference was explained by Bailey as most probably due to the rate of intestinal absorption and kidney excretion rates.

The oral fat tolerance test revealed a greater IR-GIP response in both the control and streptozotocin-treated animals when compared with the response to the oral glucose challenge. The streptozotocin-treated animals had a significantly greater IR-GIP response than the control animals. This suggests a loss of negative feedback of insulin on IR-GIP. Administration of exogenous insulin with the oral tests returned the IR-GIP responses of the streptozotocin-treated animals back to lower control levels. This suggests that in these animals insulin has a negative feedback not just on fat stimulated IR-GIP secretion but also on glucose stimulated secretion. Administration of exogenous insulin in the fasted state failed to modify the plasma IR-GIP levels in either streptozotocin-treated or control rats. Insulin therefore only acts under the conditions of nutrient stimulation and does not have a direct, solo effect on K cell secretion.

The tissue extraction and analysis revealed that the hyperphagia of the streptozotocin-treated animals caused an increase in small intestinal weight. This was most likely a secondary effect due to the increased food intake. The intestinal content of IR-GIP was significantly increased and the actual concentration of IR-GIP was
maintained in spite of the large increase in intestinal weight. The reason for this might be speculated to be the increased food intake causing increased nutrient stimulation of the K cell. IR-GLP-1(7-36)amide however was significantly increased both in terms of content and concentration. This may also be due to increased nutrient stimulation of the L cell as a result of the increased food consumption. Alternatively the deficiency of insulin in the streptozotocin-treated animals and the loss of body weight in these animals means that they were malnourished. Other products of the proglucagon hormone have been implicated to be involved in maintenance of the intestinal mucosa. Malnutrition may well have caused deleterious changes in the intestine and so the increase in intestinal GLP-1(7-36)amide could be a result of an increased demand for another product of the same prohormone. Interestingly, Kreymann et al. (1988) reported an insignificant increase in small intestinal GLP-1(7-36)amide in streptozotocin-diabetic rats 38 days post streptozotocin administration. The reason for this discrepancy is unknown.
4.3 The Effects of Autoimmune Induced Insulin Deficiency on Intestinal Entero-Endocrine Function in the Bio-Breeding (BB) Rat

4.3.1 Introduction

In 1974, spontaneous diabetes was discovered in a colony of rats maintained at Bio-Breeding Laboratories Ltd. Insulin deficiency in these animals results from massive B cell destruction mediated by an autoimmune process. The syndrome resembles human IDDM having its onset during the growth years and a similar pancreatic pathology (Bone et al., 1991). Features of the syndrome include weight loss, glucosuria, hyperglycaemia and hypoinsulinaemia. Unless exogenous insulin is administered fatalities result from severe hyperglycaemia and ketoacidosis (Nakhooda et al., 1977).

The intestinal entero-endocrine function of these animals has not been reported. The aim of this study was therefore to measure intestinal IR-GIP and IR-GLP-1(7-36)amide in these animals.

4.3.2 Methods

4.3.2.1 Animals

Eighteen BB(S) rats (14 males and 4 females) were obtained from Dr. A. Bone, Dept. of Medicine, University of Southampton. Animals were nine months of age upon arrival at Surrey and so their diabetes was of approximately six months' duration at this stage. Animals were caged in four groups of n=4 or 5, and maintained under normal laboratory conditions as described in Section 2.1, with food and water supplied ad libitum. Food intake and body weight were monitored over 24 hour periods. The four groups of rats were allocated different insulin therapies. All test groups received
exogenous insulin therapy (Ultratard Insulin, Novo, Denmark) at a dose (ranging from 2.8-3.8U/day) sufficient to maintain or slightly increase body weight. This was achieved by regular monitoring of body weight and both urine and plasma for glucose and ketone body concentrations. One group of males (n=4) received their insulin therapy daily throughout the experiment. Another group of males (n=4) received the same dose of exogenous insulin but their insulin treatment was discontinued 4 days prior to the end of the experiment. The group of females (n=4) also had their insulin therapy withdrawn 4 days prior to the end of the experiment. The final group of males (n=5) who were genotyped as BB(R) i.e. resistant to the autoimmune destruction of the B cells, were utilised as a control group. All test groups therefore had been diabetic for 9 months at the time of death.

4.3.2.2 Experimental Procedure

All animals were killed by cervical dislocation after 3 months of experimental monitoring. Their small intestines were excised, cleaned of extraneous material and treated as described previously (Section 2.4.2).

This thesis reports only food intake and body weight measurements and the GIP and GLP-1(7-36)amide content of the small intestines. Body weight and food intake results were monitored in collaboration with Dr. C. Barnett.

4.3.3 Results

4.3.3.1 Body Weight, Food Intake and Plasma Glucose

Mean body weight of the four study groups is summarised in Figure 4.3.1.
Body weights were similar in all male groups. Female BB(S) rats weighed significantly less than the male rats as expected.
The BB rats were clearly not hyperphagic, all test groups consumed significantly less food than the control animals.

Plasma glucose levels were of the order of 14-18 mmol/l in the insulin treated BB rats, when measured immediately before insulin injection. In those BB rats whose insulin therapy was withdrawn the plasma glucose levels rose from 15 to 30 mmol/l by day 4, indicating severe hyperglycaemia.

4.3.3.2 Small Intestinal Entero-Endocrine Function

Small intestinal measurements were expressed on a g/wet weight basis.
**Figure 4.3.3** Small Intestinal Wet Weight of BB Rats with and without Insulin Therapy

Values are expressed as a mean±SEM for groups of 4-5 rats.

- □ Control
- △ BB-Insulin Treated, Male
- □ BB-Insulin Withdrawn, Male
- ○ BB-Insulin Withdrawn, Female

Actual intestinal wet weights were very similar between the four study groups.
Figure 4.3.4 Small Intestinal IR-GIP of BB Rats with and without Insulin Therapy

Values are expressed as a mean±SEM for groups of 4-5 rats. Symbol, (0), indicates a value significantly different from the female and control study groups, 0=p<0.05.

Total Extractable IR-GIP
- Control
- BB-Insulin Treated, Male
- BB-Insulin Withdrawn, Male
- BB-Insulin Withdrawn, Female

Intestinal IR-GIP Concentration
- Control
- BB-Insulin Treated, Male
- BB-Insulin Withdrawn, Male
- BB-Insulin Withdrawn, Female

Total extractable IR-GIP content of the small intestines from insulin-treated BB rats was significantly greater than that of the control animals. The actual concentration of IR-GIP in the intestines was similar for all four groups.
Small Intestinal IR-GLP-1(7-36)amide of BB Rats with and without Insulin Therapy

Values are expressed as a mean±SEM for groups of 4-5 rats.

**Total Extractable IR-GLP-1(7-36)amide**

There were large variations in both the content and concentration of IR-GLP-1(7-36)amide in the small intestines of all four groups studied. No significant differences were observed between the groups.
4.3.4 Discussion

The diabetic syndrome of BB rats has been characterised by Nakhooda et al. (1977). These animals show many similarities with juvenile onset diabetes (insulin dependent diabetes) in man. In both situations there is a genetic predisposition to development of the disease and the onset of visible symptoms is abrupt, often corresponding with the time of puberty. Features of this diabetic syndrome in both man and rats are weight loss, glycosuria, hyperglycaemia and hypoinsulinaemia with subsequent ketoacidosis which often proves fatal unless exogenous insulin is given. In the case of both BB rats and man, the onset of the disease is preceded by insulitis. In clinical studies, only once has IDDM been associated with increased plasma GIP levels (Creutzfeldt et al., 1988). More frequently a reduced GIP response to nutrient stimulation has been reported in insulin dependent diabetic patients (Reynolds et al., 1978; Krarup et al., 1983; Krarup et al., 1985). In the BB rats, the only notable difference was that rats maintained on insulin therapy exhibited an increase in total intestinal GIP. No other changes in small intestinal incretin content were observed.

BB rats are glucose intolerant due to their severe hypoinsulinaemia. Initially it was believed that diabetic BB rats were hyperphagic, as is the case in streptozotocin diabetic rats. This however was not the case in the animals studied, due to their prolonged insulin treatment, even in those animals from whom the insulin treatment was withdrawn hyperphagia had not had time to become established. The chemically induced diabetes of streptozotocin diabetic rats therefore shows differences to the autoimmune syndrome of BB rats. Streptozotocin diabetes does not produce ketoacidosis and so exogenous insulin therapy is not essential to the survival of these rats. Small intestinal IR-GIP and IR-GLP-1(7-36)amide content were significantly increased in streptozotocin-treated animals contrasting with the lack of change in the BB rats. The streptozotocin-treated animals were hyperphagic and hypoinsulinaemic (31-
36 days duration) in contrast to the normal level of food consumption, insulin treatment and short term hypoinsulinaemia of the BB rats.

4.4 Conclusions

All three studies in this Chapter have investigated entero-endocrine function under pathologically altered insulin homeostasis conditions.

The insulinoma study, a condition of severe hyperinsulinaemia, revealed that hyperphagia contributed to elevated plasma IR-GIP concentrations. Insulinomas suppress normal B cell function in the pancreas by production of excessive levels of circulating insulin. The results therefore are believed to be due to the effect of exogenous stimuli on the insulinoma directly. The ability of GIP to influence insulin secretion from the tumour under very modest hyperglycaemic conditions indicates that although the insulinoma is self-governing it can be influenced by external stimuli.

The streptozotocin and BB rat studies, investigated entero-endocrine function under hypoinsulinaemic conditions. The BB rats were not hyperphagic, due to their recent or continuing insulin treatment, and there were clear differences between this form of spontaneous IDDM and that induced experimentally with streptozotocin. The streptozotocin study clearly demonstrated that the IR-GIP response of insulin-deficient animals to oral nutrients could be suppressed by simultaneously administered exogenous insulin. Long-term insulin therapy as administered to the BB rats and the absence of hyperphagia produced a normal intestinal IR-GIP and IR-GLP-1(7-36)amide concentration in the small intestine.

In the insulinoma study, hyperphagia of 6 days' duration maintained the small intestinal content of IR-GIP and IR-GLP-1(7-36)amide in the presence of a severe hyperinsulinaemia which has been shown by previous workers to suppress K cell
entero-endocrine secretion. Insulin resistance of the K cell may well be responsible for this. In the streptozotocin study, the hyperphagia was not so severe but was maintained over a longer period of time (31 days). In these animals, there was an increase in IR-GIP and IR-GLP-1(7-36)amide content of the small intestine. This was believed to be the result of maintained hyperalimentation and depletion of insulin resulting in loss of a suppressive influence on K cell secretion.

Both the streptozotocin and insulinoma studies support a strong relationship between hyperphagia and elevated plasma IR-GIP concentrations. These studies also illustrate the important influence of insulin feedback on GIP secreting K cell function and possibly also on L cell function. In contrast to human work, insulin appears to suppress not only fat stimulated GIP secretion but also carbohydrate stimulated GIP secretion in the rat.
Chapter 5.0

ACUTE EFFECTS OF GIP AND GLP-1(7-36)AMIDE ON INSULIN SECRETION IN OBESE HYPERGLYCAEMIC (oh/oh) MICE
5.1 Introduction

The origins and characteristics of the obese (ob/ob) mouse have been described by Bailey et al. (1982). This obese hyperglycaemic syndrome in mice is a recognised and widely utilised animal model of non-insulin dependent diabetes mellitus and obesity (Bailey & Flatt, 1986). In the homozygous condition, ob/ob mice exhibit an age-related development of hyperphagia, obesity, hyperinsulinaemia and hyperglycaemia; the severity of the symptoms being influenced by the genetic background.

The evidence linking diet and the entero-insular axis with obesity-diabetes syndromes of rodents was reviewed in Section 1.6.2. Obese mice have been observed to exhibit a generalised entero-endocrine cell hyperplasia (Polak et al., 1975; Best et al., 1977) and exaggerated plasma IR-insulin responses have been observed to GIP, glucagon and many other endocrine secretory products of the gastro-intestinal tract (Flatt & Bailey, 1982; Flatt & Bailey, 1987; Flatt et al., 1984; Bailey & Flatt, 1984).

At present the sensitivity of the GLP-1(7-36)amide assay limits its use in plasma measurements of small laboratory rodents. Previous chapters have reported increased intestinal GLP-1(7-36)amide levels in pregnancy, streptozotocin diabetes and during modified neonatal feeding. In contrast, in situations of insulinoma and lactation no changes were observed. The parent molecule GLP-1(1-37) has been shown to have barely any effects on insulin secretion in ob/ob mice (Bailey & Flatt, 1987). The rationale for this study was to evaluate the involvement of GLP-1(7-36)amide in the entero-insular axis and in the condition of hyperinsulinaemia. For this reason ob/ob mice were used as the chosen animal model, hyperinsulinaemia being a prominent feature of this syndrome. In addition the potency of GLP-1(7-36)amide was compared to that of GIP in order to assess whether GLP-1(7-36)amide is likely to be as potent an incretin in rodents as the evidence suggests it is in humans (Kreymann et al., 1987).
Hyperplasia of intestinal GIP secreting K cells has been reported in ob/ob mice and appears to result from hyperalimentation and impaired suppression of GIP secretion by insulin (Flatt et al., 1983a; 1984). Intestinal enteroglucagon as determined by non-specific glucagon antisera has also been reported to be increased in ob/ob mice (Flatt et al., 1983b, Flatt et al., 1983c). Intestinal entero-endocrine function was therefore assessed in this study to compare the changes in GIP secreting K-cell function with any changes in GLP-1(7-36)amide secreting L-cell function.

Pancreatic processing of proglucagon has been investigated in porcine, bovine, human and rat pancreas. The C-terminal proglucagon fragment secreted from porcine and human pancreas (Orskov et al., 1986; Orskov et al., 1987a; Kreymann et al., 1987), is further processed in rats (Shima et al., 1987; Manaka et al., 1987) giving a major peak on gel filtration identical to that of synthetic GLP-1(7-37). The pancreatic processing of ob/ob mice was investigated by means of gel filtration to determine if the C-terminal proglucagon was processed further in these animals.

5.2 Methods

Obese hyperglycaemic ob/ob mice (n=12, body weight 75-85g) from the colony maintained at the University of Aston in Birmingham were kindly donated by Dr. C.J. Bailey. Homozygous lean mice (+/+, n=11, body weight 40-50g) were used as controls. The origin of this colony has been described in detail elsewhere (Flatt & Bailey, 1981). The Aston colony is a closed non-inbred colony on a mixed genetic background.

Animals upon arrival at Surrey were caged in groups of 5-6, with an equal distribution of the sexes between the groups and under the standard conditions outlined in Section 2.1. Food intake and body weight were monitored over consecutive 24 hour
periods prior to the start of the acute tests. Animals were fasted for 18 hours immediately prior to the acute tests.

In the first provocative test, following an 18 hour fast, porcine GIP(1-42) was administered intraperitoneally at a dose of 40µg/kg (8.04mmol/kg) in a vehicle of glucose (33.1kJ/kg body weight equivalent to 2g/kg) or saline (0.9% w/v). The volume of the dose was standardised at 5ml/kg body weight. Blood samples (50µl) were obtained from the cut tail tip of the mice immediately prior to and at 5, 15 and 30 minutes following injection. Determinations of plasma glucose (5µl for obese mice, 10µl for lean mice) and IR-insulin (10µl for obese mice and 20µl for lean mice), were performed on individual samples as described previously (Sections 2.7 and 2.8).

Seven days later the above experimental procedure was repeated using human GLP-1(7-36)amide (Bachem, Switzerland). The human GLP-1(7-36)amide was used at an equimolar dose to porcine GIP of 33µg/kg (8.04mmol/kg body weight) in freshly prepared saline or glucose vehicles. Finally, seven days later the experiment was repeated for a third and final time using fresh batches of the vehicles alone.

Animals were culled by cervical dislocation. Small intestines and whole pancreas were dissected from the animals as described previously and individually frozen to -20°C prior to extraction into acid ethanol (Section 2.4.2). Small intestinal extracts were subsequently assayed for IR-GIP and IR-GLP-1(7-36)amide as described previously (Sections 2.5 and 2.6).

In addition, pooled small intestinal and pooled pancreatic extracts were dried down and reconstituted in gel filtration buffer. These extracts were then subjected to gel filtration and the fractions collected, dried down and assayed for IR-GLP-1(7-36)amide content using both the G2 and the 2135 antisera. The procedures involved were outlined fully in Section 2.6.5.
5.3 Results

5.3.1 Body Weight and Food Intake

Mean body weights of lean and ob/ob mice were monitored immediately prior to each provocative test. The results are illustrated in Figure 5.1.1.

**Figure 5.1.1** Body Weight of Obese Hyperglycaemic (ob/ob) and Lean (+/+)
Mice

Values are expressed as mean±SEM for groups of 11-12 mice. Symbol (**) indicates values significantly different from +/+ mice when assessed by Student's unpaired t-tests, **=p<0.01.

It can clearly be seen that the ob/ob mice were approximately double the weight of the lean mice. Within the two groups body weights continued to be static throughout the duration of the study.
The obese mice were significantly hyperphagic throughout the duration of the study.

5.3.2 Acute Provocative Tests

5.3.2.1 Lean Mice

The results of administration of the test solutions to lean mice are summarised graphically in Figure 5.1.3 and 5.1.4.
Figure 5.1.3  The Effects of Acute Intraperitoneal Administration of Porcine GIP or Human GLP-1(7-36)amide in Saline on the Plasma Glucose and IR-Insulin Response of Homozygous Lean (+/+ ) Mice

Values are expressed as mean±SEM for groups of 5-6 animals. Saline refers to the vehicle only.

Plasma Glucose

In a saline vehicle, neither GIP nor GLP-1(7-36)amide had any significant effects on glucose homeostasis or plasma insulin concentrations.
The Effects of Acute Intraperitoneal Administration of Porcine GIP or Human GLP-1(7-36)amide in Glucose on the Plasma Glucose and IR-Insulin Response of Homozygous Lean (+/+ ) Mice

Values are expressed as mean±SEM for groups of 5-6 animals. Glucose refers to the vehicle only. Symbols (*, **) indicate values significantly different between the two groups when assessed by one way analysis of variance and Duncan's range test, *=p<0.05; **=p<0.01.

In a glucose vehicle, both GIP and GLP-1(7-36)amide were seen to significantly improve glucose tolerance and insulin secretion. Integrated plasma glucose and IR-insulin responses in the presence of glucose are tabulated in Table 5.1.
The integrated results confirm the improvement in glucose tolerance and insulin secretion in the presence of GIP and GLP-1(7-36)amide. GIP caused over a four-fold increase in mean plasma insulin concentration and GLP-1(7-36)amide a five-fold increase. There was no significant difference in the degree of stimulation by the two hormones.

Table 5.1  **Integrated Plasma Glucose and IR-Insulin Responses of Lean Homozygous (+/+). Mice in Response to Intraperitoneal Administration of Porcine GIP or Human GLP-1(7-36)amide**

Values are expressed as mean±SEM for groups of 5-6 animals. Symbol (**) indicates a value significantly different from the two other groups when assessed by one way analysis of variance and Duncan's range test, **=p<0.01.

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<tr>
<th>Nature of Provocative Test</th>
<th>Integrated Plasma Glucose Response (mmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-Insulin Response (ng/ml).hr⁻¹</th>
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<tr>
<td>IP Glucose</td>
<td>53.13 ± 3.88 **</td>
<td>2.75 ± 1.24 **</td>
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<td>IP Porcine GIP and Glucose</td>
<td>31.46 ± 2.35</td>
<td>11.76 ± 1.80</td>
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<td>IP Human GLP-1(7-36)amide and Glucose</td>
<td>22.27 ± 3.24</td>
<td>14.03 ± 2.05</td>
</tr>
</tbody>
</table>

5.3.2.2 **Obese Mice**

The results of administration of the test solutions to obese mice are summarised graphically in Figure 5.1.5 and 5.1.6.
Figure 5.1.5 The Effects of Acute Intraperitoneal Administration of Porcine
GIP or Human GLP-1(7-36)amide in Saline on the Plasma
Glucose and IR-Insulin Response of Obese Hyperglycaemic
(ob/ob) Mice

Values are expressed as mean±SEM for groups of 5-6 animals. Saline refers
to the vehicle only.

As with the lean mice, at basal plasma glucose concentrations neither GIP nor
GLP-1(7-36)amide had any significant effect on plasma IR-insulin levels.
Figure 5.1.6  The Effects of Acute Intraperitoneal Administration of Porcine GIP or Human GLP-1(7-36)amide in Glucose on the Plasma Glucose and IR-Insulin Response of Obese Hyperglycaemic (ob/ob) Mice

Values are expressed as mean±SEM for groups of 5-6 animals. A symbol (*) indicates a value significantly different from the two other groups when assessed by one-way analysis of variance and Duncan’s range test, *=p<0.05. A symbol (o) indicates a value significantly different from the control group when assessed by one-way analysis of variance and Duncan’s range test, o=p<0.05.

**Plasma Glucose**

- Glucose
- GIP + Glucose
- GLP-1 + Glucose

**Plasma IR-Insulin**

- Glucose
- GIP + Glucose
- GLP-1 + Glucose
In the presence of glucose, both GIP and GLP-1 significantly improved glucose tolerance and increased insulin secretion. Since both hormones were administered at equimolar doses it can be seen from the integrated insulin responses (Table 5.2) that the two hormones were approximately equipotent in their actions. Porcine GIP caused an average 2.6 fold increase in plasma insulin concentration and GLP-1(7-36)amide a 3.4 fold increase.

Table 5.2 Integrated Plasma Glucose and IR-Insulin Responses of Obese (ob/ob) Mice in Response to Intraperitoneal Administration of Porcine GIP or Human GLP-1(7-36)amide

Values are expressed as mean±SEM for groups of 5-6 animals. Symbol (**) indicates a value significantly different from the two other groups when assessed by one way analysis of variance and Duncan's range test, **=p<0.01.

<table>
<thead>
<tr>
<th>Nature of Provocative Test</th>
<th>Integrated Plasma Glucose Response (mmol/l).hr⁻¹</th>
<th>Integrated Plasma IR-Insulin Response (ng/ml).hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP Glucose</td>
<td>83.79 ± 3.37 **</td>
<td>37.72 ± 9.47 **</td>
</tr>
<tr>
<td>IP Porcine GIP and Glucose</td>
<td>55.21 ± 4.01</td>
<td>97.02 ± 8.00</td>
</tr>
<tr>
<td>IP Human GLP-1(7-36)amide and Glucose</td>
<td>56.92 ± 4.78</td>
<td>127.40 ± 7.47</td>
</tr>
</tbody>
</table>

5.3.3 Small Intestinal Entero-Endocrine Function

Small intestinal wet weights of lean and ob/ob mice are summarised in Figure 5.1.7.
Figure 5.1.7  

**Small Intestinal Wet Weight of Lean (+/+) and Obese (ob/ob) Mice**

Values are expressed as mean±SEM for groups of 8 lean and 10 obese mice. Symbol (**) indicates a value significantly different from lean animals when assessed by Student's unpaired t-test, **=p<0.01.

The actual intestinal wet weight of ob/ob mice was significantly increased in comparison to the lean animals.
Small Intestinal IR-GIP of Lean (+/+) and Obese (ob/ob) Mice

Values are expressed as mean±SEM for groups of 8 lean and 10 obese mice. Symbol (**) indicates a values significantly different from lean animals when assessed by Student's unpaired t-test, **=p<0.01.

Obese hyperglycaemic (ob/ob) mice showed a highly significant increase in small intestinal content and concentration of IR-GIP when compared to lean (+/+) mice.
Figure 5.1.9 Small Intestinal IR-GLP-1(7-36)amide of Lean (+/+) and Obese (ob/ob) Mice

Values are expressed as mean±SEM for groups of 8 lean and 10 obese mice. Symbol (**) indicates a value significantly different from lean animals when assessed by Student's unpaired t-test, **=p<0.01.

Total Extractable IR-GLP-1(7-36)amide

Intestinal IR-GLP-1(7-36)amide Concentration

The changes apparent in intestinal IR-GIP in obese mice were also present in IR-GLP-1(7-36)amide content and concentration. The actual content and concentration of IR-GLP-1(7-36)amide was significantly increased in obese mice.
5.3.4 Gel Filtration of Obese (ob/ob) Small Intestine and Pancreatic Extracts

5.3.4.1 Pancreatic Tissue Extracts

The results of measuring IR-GLP-1(7-36)amide in pancreatic extracts of obese mice using both the G2 and the 2135 antisera are illustrated in Figure 5.1.10.

Figure 5.1.10 Total Pancreatic IR-GLP-1(7-36)amide Content of Obese (ob/ob) Mice

Values are expressed as mean±SEM for groups of 10 obese mice. IR-GLP-1(7-36)amide content was measured with the G2 and the 2135 antisera. Symbol (**) indicates a value significantly different from the result with the other antiserum when assessed by Student's paired t-test, **=p<0.01.

It can clearly be seen that the non-specific 2135 antiserum detected more IR-GLP-1(7-36)amide than the G2 antiserum. Gel filtration of extracts was carried out to determine the molecular weight profile of the immunoreactive material. The results of the gel filtration are illustrated in Figures 5.1.11 and 5.1.12.
Figure 5.1.11  Gel Filtration Profile of Pancreatic IR-GLP-1(7-36)amide Extracts of Obese (ob/ob) Mice With Antiserum 2135

Vo is the void volume of the column; CC is the elution maximum of cytochrome C; GLP-1(7-36)amide is the elution maximum of a human standard preparation previously run through the column; B12 is the elution maximum of cyanocobalamin. Kav was defined in Chapter 2, 2.6.6

Pancreatic Extract 1

Pancreatic Extract 2

Pancreatic Extract 3
Figure 5.1.12 Gel Filtration Profile of Pancreatic IR-GLP-1(7-36)amide Extracts of Obese (ob/ob) Mice With Antiserum G2

Vo is the void volume of the column; CC is the elution maximum of cytochrome C; GLP-1(7-36)amide is the elution maximum of a human standard preparation previously run through the column; B12 is the elution maximum of cyanocobalamin. Kav was defined in Chapter 2, 2.6.6.
Gel filtration of obese mouse pancreatic extracts by Sephadex G-50 and
detection of immunoreactivity by Antiserum 2135 revealed a major form of GLP-1-like
immunoreactivity at the position of synthetic GLP-1(7-36)amide (Kav 0.477-0.489).
Antiserum G2 also detected an immunoreactive peak at this position, but the peak was
far smaller than that detected by Antiserum 2135. In addition two minor
immunoreactive peaks were detected by Antiserum 2135, one being located close to the
void volume and the other at 0.30 Kav, corresponding to a molecular weight of
approximately 10000 daltons. Both of these minor peaks were absent from detection
by Antiserum G2. Basal immunoreactivity levels were also detected to be higher when
measured by Antiserum 2135.

5.3.4.2 Small Intestinal Tissue Extracts

The results of measuring IR-GLP-1(7-36)amide in gel filtration fractions of
small intestinal extracts of ob/ob mice using both the G2 and the 2135 antisera are
illustrated in Figures 5.1.13 and 5.1.14.

Both Antiserum 2135 and G2 detected a single peak of immunoreactivity which
eluted at the position of synthetic GLP-1(7-36)amide. The concentrations of IR-GLP-
1(7-36)amide detected by the two antisera were very similar. Antiserum 2135 detected
approximately 10-15% more immunoreactivity per fraction tube than antisera G2.
Figure 5.1.13  Gel Filtration Profile of Small Intestinal IR-GLP-1(7-36)amide Extracts of Obese (ob/ob) Mice With Antiserum 2135

Vo is the void volume of the column; CC is the elution maximum of cytochrome C; GLP-1(7-36)amide is the elution maximum of a human standard preparation previously run through the column; B12 is the elution maximum of cyanocobalammin. Kav was defined in Chapter 2, 2.6.6.

**Intestinal Extract 1**

**Intestinal Extract 2**
Figure 5.1.14 Gel Filtration Profile of Small Intestinal IR-GLP-1(7-36)amide Extracts of Obese (ob/ob) Mice With Antiserum G2

Vo is the void volume of the column; CC is the elution maximum of cytochrome C; GLP-1(7-36)amide is the elution maximum of a human standard preparation previously run through the column; B12 is the elution maximum of cyanocobalamin. Kav was defined in Chapter 2, 2.6.6.

Intestinal Extract 1

Intestinal Extract 2
5.4 Discussion and Conclusions

The obese hyperglycaemic (ob/ob) mice demonstrated a marked hyperphagia and increased body weight in comparison to the lean (+/+) control animals. Fasting the ob/ob animals for 18 hours lowered their plasma glucose levels and reduced their hyperinsulinaemia but the fast was insufficient to return their plasma insulin and glucose profile back to the normal control values.

The ob/ob mouse has been shown to exhibit markedly raised plasma GIP concentrations (Flatt et al., 1983a). This increase in plasma GIP contributes to the hyperinsulinaemia of these animals (Flatt et al., 1984). An exaggerated insulin secretion in response to exogenous porcine GIP has previously been reported in Aston ob/ob mice (Flatt et al., 1984).

In the present study, acute administration of GIP or GLP-1(7-36)amide in saline revealed the importance of glucose in modulating the responsiveness of the B cells to the two hormones. Both GIP and GLP-1(7-36)amide showed no effects on glucose homeostasis or insulin secretion in the presence of normoglycaemia. In the presence of a modest hyperglycaemia both GIP and GLP-1(7-36)amide were seen to be capable of augmenting insulin secretion in both the lean and the ob/ob animals. This confirms the observation of Fridolf et al. (1990b) that GLP-1(7-36)amide stimulates insulin secretion in mice. The potency of both hormones when assessed by the magnitude of the increase in insulin stimulation was greatest in the lean animals. Since obese mice are known to demonstrate increased circulating levels of GIP possible down regulation of the GIP receptors on the B cells or reduced storage capacity for insulin within the B cells may account for the reduced sensitivity of their B cells.

The infusion of exogenous GLP-1(7-36)amide in man has been shown to elicit a greater insulin response than GIP at post prandial concentrations (Kreymann et al.,...
In the present study equimolar doses of GIP and GLP-1(7-36)amide were administered at a dose known to produce a physiological increase in plasma IR-GIP (Flatt et al., 1984). In man GLP-1(7-36)amide has been shown to be four times as potent as GIP on a molar basis (Kreymann et al., 1987). This difference in potency was not detectable in this present study. The half life of exogenous GIP in ob/ob mice is known to be short, approximately 15 minutes (Flatt et al., 1984) as opposed to 30 minutes in humans (Kreymann et al., 1987). The half life of GLP-1(7-36)amide in humans is known to be significantly shorter than that of GIP (Kreymann et al., 1987). In rats the half life of GLP-1(7-36)amide has been reported to be approximately 4.5 minutes (Ruiz-Grande et al., 1990) supporting a faster turnover of this hormone compared with GIP. It can be speculated that the same is also true for ob/ob mice and if this is so increased turnover of GLP-1(7-36)amide may account for the similar potency of the two gut hormones.

Hyperplasia of intestinal mucosa K cells has been noted in ob/ob mice (Flatt et al., 1983a). Increased intestinal GIP concentration has also been reported and is believed to result in part from hyperalimentation and impaired suppression of GIP secretion by insulin (Flatt et al., 1983a, 1984). In the present study the increase in intestinal GIP content and concentration was noted. Similar results were also obtained for IR-GLP-1(7-36)amide and this raises the question of whether the changes in IR-GLP-1(7-36)amide are also partially the result of hyperalimentation and impaired suppression by insulin or whether another mechanism is operating on the control of L cell secretion. The increase in intestinal IR-GLP-1(7-36)amide in ob/ob mice would suggest that the plasma levels of this hormone are also likely to be elevated. Elevated plasma levels of GIP and GLP-1(7-36)amide may therefore contribute to the severe hyperinsulinaemia of ob/ob mice by stimulation of the B cell. Additionally in rats receiving prolonged nutrient infusion, hyperinsulinaemia was promoted in part by a suppression of insulin receptor binding. Prolonged exogenous GIP infusion further...
suppressing receptor activity (Baer and Dupré, 1985). This may therefore offer another mechanism by which GIP promotes the hyperinsulinaemia of ob/ob mice.

The A cells of the pancreas and the L cells of the intestinal mucosa have identical mRNA's coding for preproglucagon. However it is believed that these cells differ in their post translational enzymic processing (Mojsov et al., 1986). It has been reported that most of the GLP-1-like immunoreactivity in human pancreatic extracts co-elutes with GLP-2-like immunoreactivity in a 10000 dalton molecular weight fragment, the so called C-terminal fragment (Uttenthal et al., 1985; George et al., 1985). These results have been confirmed in porcine pancreas extracts (Holst et al., 1987). In rats however the major fraction of GLP-1-like immunoreactivity elutes at the position of synthetic GLP-1(1-37), (Shima et al., 1987; Manaka et al., 1987). The results from the gel filtration of ob/ob mice pancreatic extracts suggest that these mice, like rats, show further processing of the C-terminal fragment. The GLP-1-like immunoreactivity measured is mainly of the 1-37 or even the 7-37 form as only a small proportion cross reacts with the G2 antisera which is specific for the 36-amide forms of the molecule. The function of the GLP-1-like immunoreactivity released from the pancreas is unknown but the level is far lower than that detected in the intestine.

In the intestine of ob/ob mice the major form of GLP-1 like immunoreactivity is the 36-amide form. Gel filtration of ob/ob intestines and detection of filtration products with non-specific glucagon antiserum has revealed that ob/ob mice intestine contains large amounts of low molecular weight glucagon like immunoreactivity (GLI) (Flatt et al., 1983b; Flatt et al.., 1983c). This agrees with the high concentration of IR-GLP-1(7-36)amide detected in ob/ob intestines in this study.

In conclusion GLP-1(7-36)amide is a potent incretin in mice. Demonstration of its release in response to nutrient stimulation is therefore necessary to support the potential incretin role of endogenous GLP-1(7-36)amide in rodents.
Chapter 6.0

EFFECTS OF VARIOUS SUGARS ON THE IN-VITRO SECRETION OF IR-GIP AND IR-GLP-1(7-36)AMIDE
6.1 Introduction

GLP-1(7-36)amide is a polypeptide secreted from mucosal L-cells located mainly in the distal small intestine and colon (Kreymann et al., 1988). IR-GLP-1(7-36)amide has been shown to be released into the plasma in response to an oral glucose load or a mixed meal and to stimulate insulin release (Kreymann et al., 1987). GLP-1(7-36)amide has therefore been proposed to be an important physiological incretin in man (Kreymann et al., 1987). An understanding of the mechanism of stimulus-secretion coupling for this hormone is an important step in the demonstration that this hormone is a true incretin that qualifies the criteria outlined in Section 1.2.3.

GIP is an acknowledged incretin in man, rodents and other animals (Jones et al., 1987; Kreymann et al., 1987; Flatt et al., 1984). It is secreted by mucosal K-cells located primarily in the proximal small intestine (Bloom and Polak, 1981). An intestinal perfusion technique has revealed that IR-GIP secretion is stimulated by actively absorbed sugars (Sykes et al., 1980) and fats (Kwasowski et al., 1985). A perfusion technique for the assessment of IR-GLP-1(7-36)amide stimulus-secretion coupling would be very difficult in rodents with the very low circulating levels of this hormone and the necessarily large amounts of plasma required to measure the hormone. A recent intestinal perfusion study in dogs revealed that IR-GLP-1(7-36)amide secretion could be stimulated by certain saccharides via a "glucose sensor" without absorption of the sugar being a prerequisite for secretion (Shima et al., 1990).

The aim of this study was to investigate the effects of sugars with different mechanisms of absorption on the secretion of GLP-1(7-36)amide by using an in-vitro system. As a control measurement, to demonstrate the suitability of this system for such a procedure, IR-GIP secretion to the various stimuli was also evaluated. The
advantage of an in-vitro system, apart from the cost, is that the prepared intestine is completely denervated and so the mechanism of stimulation is solely hormonal.

6.2 Methods

6.2.1 Animals and their Housing Conditions

Fifty two male, Wistar albino rats of 200-250g body weight were used in this study. The origin of these animals and their housing conditions were described in Section 2.1. All animals were fasted for 18 hours (overnight), prior to the experimental procedure.

6.2.2 Experimental Design

6.2.2.1 Reagents

All chemicals unless otherwise stated were obtained from BDH Chemicals Ltd., Poole, Dorset.

Krebs Saline:

Krebs saline perfusion medium was used as the perfusate and also as a control incubation solution. The perfusion fluid consisted of a mixture of 0.154M solutions of the following salts mixed in the volumes indicated in the right hand column of Table 6.1.
Table 6.1 The Constituents of Krebs Saline Perfusion Medium

<table>
<thead>
<tr>
<th>Salt Solution</th>
<th>Volume to be Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.154M (0.90%) NaCl</td>
<td>100</td>
</tr>
<tr>
<td>0.154M (1.15%) KCl</td>
<td>4</td>
</tr>
<tr>
<td>0.154M (2.11%) KH₂PO₄</td>
<td>1</td>
</tr>
<tr>
<td>0.154M (3.82%) MgSO₄</td>
<td>1</td>
</tr>
<tr>
<td>0.154M (1.30%) NaHCO₃</td>
<td>21</td>
</tr>
</tbody>
</table>

The perfusion solution was adjusted to pH 7.2-7.3 and gassed with 95% O₂ /5% CO₂ for a minimum of 20 minutes prior to use.

Sugar Solutions Tested:

The following test solutions were freshly prepared in Krebs saline perfusion medium;

1 25mM β-D-Glucose
2 25mM β-D-Galactose
3 25mM β-D-Fructose
4 25mM Lactose
5 25mM Sucrose
6 25mM Maltose
7 10mM β-D-Glucose
8 25mM β-D-Glucose + 2.5mM Phloridzin (Sigma Chemical Co. Ltd., London)

The control was the saline perfusion medium alone. Each sugar solution was gassed with 95% O₂ /5% CO₂ for a minimum of 10 minutes prior to use. All solutions were maintained at approximately 4°C prior to incubation with the everted gut sacs.
6.2.2.2 Experimental Procedure

The everted gut sac technique of Wilson & Wiseman (1954), was used for this study. This method was reviewed by Madge (1975).

Rats were killed by cervical dislocation and the abdomen opened by midline incision. The small intestine was cut at the ileo-caecal junction and the final 15cm length of distal small intestine removed. The mesentery was carefully removed from the intestine. The intestinal lumen was flushed out with 0.9% NaCl to clean it of adhering food debris. The isolated intestine was placed in a dish of ice cold gassed perfusion medium. All subsequent manipulations of the tissue were carried out at 4°C.

A 12cm glass rod with an indentation 1cm from one end was gently inserted into the intestine and a ligature tied around the intestine to secure it on the indentation in the rod. The intestine was carefully everted by rolling the intestine over its invaginated end. The everted intestine was then cut just below the ligature and slid off the rod into a 50cm³ flask of oxygenated perfusion medium at 4°C. The tissue was allowed to rest in this medium for 10 minutes with constant gassing.

After resting the intestine was cut into two lengths (transversely) in order to make two gut sacs. One end of an intestinal length was tied off by a thread ligature which was left untrimmed. The sac was lifted by means of this ligature in order to drain the contents of the sac. A second ligature was placed around the other end of the drained sac. Through a blunt needle fresh perfusion medium was injected until the sac was just distended. The ligature was tightened and the needle withdrawn and finally the sac was tied off.

Each prepared sac was checked for leakage and placed in an incubating flask (50cm³) containing 25mls of an incubating sugar solution or 25mls of the perfusion
medium alone (control). The flask was gassed for 2 minutes with 95% O₂/5% CO₂ and then sealed and mechanically shaken in a preheated water bath (37°C), for the required length of time. Table 6.2 gives the range of solutions, the incubation times looked at and the number of gut sacs per treatment used in this study.

Table 6.2  Protocol of the Study

<table>
<thead>
<tr>
<th>Incubation Solution</th>
<th>Incubation Time</th>
<th>Number of Gut Sacs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs Saline</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Krebs Saline</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Krebs Saline</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>25mM Glucose</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>25mM Glucose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Glucose</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>10mM Glucose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Galactose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Fructose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Maltose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Sucrose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Lactose</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>25mM Glucose +2.5mM Phloridzin</td>
<td>15</td>
<td>8</td>
</tr>
</tbody>
</table>

An incubation time of zero was achieved by preparing the sac as normal and not incubating in the shaking water bath but proceeding with the next step immediately. At the end of the incubation the sac was removed from the flask by the handling of the untrimmed ligature and washed by dipping through ice-cold perfusion medium. The sac was drained and the mucosal surface gently blotted dry on filter paper prior to weighing the gut sac. The serosal fluid was then drained out into a test tube by holding the sac vertically and snipping it immediately above the bottom ligature. The drained sac was then reweighed. Both ligatures and the excess intestinal tissue were cut off as
close as possible to the point of tie and the sac reweighed. The sac was then dried at 80°C overnight and reweighed. For each sac the serosal fluid weight, effective weight of fresh tissue and the dry weight of tissue were recorded. The serosal fluid weight was obtained from the weight of the intact sac minus the weight of the drained sac. The effective weight of fresh tissue was taken as the weight of the sac after removal of the ligatures. Dry weight of tissue was taken as the weight of fresh tissue after drying.

The serosal fluid collected was immediately aliquoted for IR-GLP-1(7-36)amide assay (50µl), and IR-GIP assay (100µl). Aprotinin 600 KIU/ml in phosphate buffer (0.04M, pH 6.5), was added to the aliquots for GLP-1(7-36)amide assay. All aliquots and excess serosal fluid was immediately frozen to -20°C and maintained at this temperature until assayed at a later date.

6.2.3 RIA Procedure

With the in-vitro gut sac method substances transferred from the incubating (mucosal) fluid to the serosal fluid (the fluid within the sac) cannot be transported away. This raised several questions about the suitability of this procedure to look at GIP and GLP-1(7-36)amide secretion; would glucose absorbed by the sac interfere with the assay procedure and what would be the effects of Krebs saline on the assay? Since the GIP and GLP-1(7-36)amide assays are virtually identical in protocol these potential problems were investigated in the GLP-1(7-36)amide assay.

The influence of glucose on the GLP-1(7-36)amide standard curve was investigated by preparing a range of GLP-1(7-36)amide standards (50-1500pmol/l), in phosphate buffer (pH6.5, 0.04M), containing glucose at concentrations of 10mmol/l, 25mmol/l, 50mmol/l and 100mmol/l. The result was that standard curves prepared in a glucose solution up to 50mmol/l were indistinguishable from the normal assay standard curve.
The influence of the perfusion medium on the GLP-1(7-36)amide assay was investigated by substituting 100µl of perfusion medium for 100µl of assay buffer in the NSB, zero and standards tubes on day 1 of the assay. The perfusion medium influenced the assay binding. The standard curves in both the GLP-1(7-36)amide and the GIP assays were therefore run in the presence of perfusion medium. In the case of the GLP-1(7-36)amide assay 50µl, and in the case of the GIP assay 100µl. In addition the GIP assay was run with a human GIP standard rather than a porcine GIP standard simply for reasons of availability. The porcine GIP assay has been shown to be unable to distinguish human GIP standards from porcine GIP standards (Dr L.M. Morgan personal communication). Serial dilutions of a gut sac serosal fluid sample showed parallelism with the standard curve obtained.

6.3 Results

6.3.1 The Influence of Incubation Time on Gut Sac IR-GIP and IR-GLP-1(7-36)amide Content

The results were calculated on a g dry weight basis. The results are summarised in Figures 6.1.1 and 6.1.2.
The Effect of Incubation Time on the Content of IR-GIP in the Serosal Fluid of Gut Sacs

A clear relationship was observed between the length of the incubation period and the amount of IR-GIP in the serosal fluid of the gut sacs incubated in a glucose solution. The amount of accumulated IR-GIP increased significantly at each incubation time for those sacs incubated in a glucose solution, (p<0.01 for 0-15 minutes and p<0.05 for 15-30 minutes). An incubation period of 15 minutes was decided upon for subsequent experiments.
No clear relationship was observed between the length of the incubation period and the content of IR-GLP-1(7-36)amide in the serosal fluid. In addition, there was no significant difference in the IR-GLP-1(7-36)amide content of the serosal fluid between those sacs incubated in the saline perfusate and those incubated in 25mM glucose.

6.3.2 The Influence of Various Sugars on Gut Sac IR-GIP Content

The results are summarised in Figure 6.1.3.
Only glucose, at both a 10mM and 25mM concentration, and galactose significantly increased IR-GIP content of the gut sacs above the control (saline) content. The addition of 2.5mM phloridzin to the 25mM glucose solution reduced the IR-GIP content of gut sacs incubated in this solution until the IR-GIP content was no longer significantly increased above the IR-GIP content of saline incubated sacs. A lactose incubation solution had least effect on IR-GIP accumulation in the serosal fluid of the gut sac.
The only significant difference was between the lactose and galactose incubated sacs. For all the other incubation solutions the amount of IR-GLP-1(7-36)amide in the serosal fluid did not vary significantly. Saline stimulated a higher mean accumulation of IR-GLP-1(7-36)amide than all the sugars with the exception of galactose. Phloridzin had no observable effect on glucose stimulation of IR-GLP-1(7-36)amide secretion.

6.4 Discussion

With an in vivo intestinal perfusion system there is an unlimited supply of oxygen (from the abdominal aorta) and energy (in the form of blood glucose and nutrients absorbed from the intestinal lumen) to the tissues. The in-vitro system is limited by the short supply of oxygen and energy available but it does have the
advantage that substances transferred across the intestinal wall cannot be transported away. The two gastrointestinal hormones of interest to this study were measured in the serosal fluid of the gut sacs, not the incubation medium. Any hormone released into the mucosal fluid (incubating solution) would be diluted below the detection limit of the assays. A study investigating the release of glucagon-like immunoreactivity (GLI), in the perfused jejunum of rats demonstrated that the eluate from the lumen of the jejunum showed only a slight increase in GLI concentration during the perfusion (Wójcikowski et al., 1985). Therefore any loss of IR-GIP or IR-GLP-1(7-36)amide into the incubating solution in this study can be assumed to be minimal.

The gut sacs utilised in this study were prepared from the terminal ileum region of the small intestine. The reason for this is that this area of the intestine is a site of high GLP-1(7-36)amide concentration as assessed by tissue extraction methods (Kreymann et al., 1988). Since the main aim of this study was to investigate GLP-1(7-36)amide secretion, an area of tissue rich in this hormone was therefore necessary. The main area of distribution of GIP is the duodenum and jejunum but in rodents the concentration of extractable IR-GIP is still high in the ileum (Ponter et al., 1990). This area of tissue was therefore suitable for the assessment of secretion of both hormones.

The stimulus-secretion coupling of the GIP secreting K cell was investigated by Sykes and colleagues (1980). They reported that the sugars glucose, galactose, maltose and sucrose, among others, could stimulate IR-GIP release. The GIP secreting monosaccharides - and in the case of disaccharides their monosaccharide hydrolysis products - were concluded to be those sugars that were actively transported across the mucosal cell brush border into the mucosal cell.

In this present study only glucose and galactose stimulated a significantly greater IR-GIP accumulation within the gut sac than saline. Glucose and galactose
share a common sodium dependent carrier protein which possesses a lower \( K_m \) for glucose than galactose (Crane, 1965; 1968). Galactose stimulated a greater mean increase in IR-GIP accumulation within the gut sac (a 4.7 fold increase), than glucose (a 4 fold increase) at the same concentration. This reflects the preference of the carrier protein. Active transport is an energy requiring process and this is why the incubation period was limited to 15 minutes for the comparison of the various sugars. The transport system was aided by the high concentration of sodium ions in the perfusion medium. Reducing the concentration of glucose in the incubation medium from 25mM to 10mM had no significant effect on the accumulation of IR-GIP in the serosal fluid. This suggests that the gut sacs were incubated in an excess glucose solution at 25mM and active transport by the carrier system was near saturation at 10mM glucose concentration. Phloridzin, a \( \beta \)-D-glucoside, competitively inhibits the sodium dependent carrier protein. Addition of phloridzin to a 25mM glucose solution significantly reduced the accumulation of IR-GIP within the serosal fluid. This confirms the findings of Sykes et al. (1980).

Hydrolysis of disaccharides to constituent monosaccharides is carried out by specific enzymes situated close to the luminal side of the brush border. The observation that insignificant accumulation of IR-GIP occurred in those sacs incubated in disaccharide solutions may be explained by the low levels of disaccharidases present in the terminal ileum. The optimal location of sugar absorptive capacity being the jejunum and proximal ileum (Alpers, 1987). Lactose was the least stimulatory disaccharide sugar investigated. This may well be because lactase levels are very low in the intestinal mucosa of the mature rat (Rubino et al., 1964). Fructose uses a separate carrier system than glucose and the observation that it failed to stimulate IR-GIP secretion is in agreement with the findings of Sykes et al. (1980).

The stimulus-secretion coupling of IR-GLP-1(7-36)amide is not so straightforward. No clear relationship between the incubating sugar solution and the amount
of IR-GLP-1(7-36)amide released was observed. The control saline perfusion medium appeared to be one of the most successful stimuli for IR-GLP-1(7-36)amide accumulation in the serosal fluid. A study investigating GLI release, by a perfusion protocol, has reported that hypotonic solutions, especially water, stimulated GLI release in the plasma (Matsuyama et al., 1981). If the saline results are ignored in the statistical evaluation of the experiment, then galactose stimulated accumulation of IR-GLP-1(7-36)amide is significantly greater (p<0.05), than the results obtained with 25mM glucose or 25mM glucose and phloridzin, or lactose or fructose in the incubation medium. Shima and co-workers (1990) reported an increased secretion of GLP-1(7-36)amide in the presence of sugars that activated a sensor without being metabolised. In the case of disaccharides the disaccharides themselves not their constituent monosaccharides stimulated GLP-1(7-36)amide release. However the standard deviation of the response of individual animals in this study was extremely large. In the present study there was a trend towards an increased IR-GLP-1(7-36)amide accumulation with increasing incubation time in the presence of glucose.

Several earlier workers investigated nutrient stimulation of GLI using non-specific N-terminal directed glucagon antisera reported GLI was modulated by a number of dietary factors including glucose and fat (Read et al., 1984; Unger et al., 1968; Ohnede et al., 1975). A study which investigated release of GLI in rats by an intestinal perfusion system did show an increase in plasma GLI, as detected by a non-specific glucagon antiserum (Wójcikowski et al., 1985). The perfusion was continuous and a biphasic GLI response was seen with peak plasma GLI concentration being recorded seventy minutes after the start of the perfusion. In contrast, Shima et al. (1990), recorded their response over fifteen minutes following a ten minute control period. The rate of perfusion was not reported by Shima et al. (1990). Glucose failed to stimulate GLI release from cultured foetal intestinal cells in the absence of a physiological concentration of insulin (Brubaker, 1987).
A recent study investigating oral nutrient stimulation of IR-GLP-1(7-36)amide secretion in man using the Surrey antisera revealed only a very modest (in the order of 10 pmol/l) and transient increase in the plasma concentration of GLP-1(7-36)amide. The fasting range of the hormone was 14-17pmol/l and following individual nutrient stimulation this maximally increased to 23±1.3pmol/l with fat (Elliott et al., 1991). These observations suggest that GIP may represent the principal component of the entero-insular axis in both man and animals.

6.5 Conclusions

The in-vitro technique of gut sacs largely confirmed the findings of workers who had used the intestinal perfusion technique to investigate stimulus-secretion coupling for GIP. The results for GLP-1(7-36)amide secretion were however not as anticipated. No sugar solution significantly altered the secretion of this hormone from the basal secretion seen with saline and the individual variation in accumulated levels in the serosal fluid was greater than the variation in GIP. The evidence presented here would suggest that possibly GLP-1(7-36)amide is not an incretin and the observation by Shima et al. (1990) that metabolism is not necessary for stimulation may suggest that a nervous mechanism of stimulation exists. In man, a peak IR-GLP-1(7-36)amide concentration is seen 30 minutes after an oral glucose load (Kreymann et al., 1987). For a hormone located mainly in the distal ileum and colon this is a very rapid response as transit time through the small intestine in fasted humans is very variable, between 30 and 140 minutes being required for complete passage through the small intestine (Caride et al., 1984).

To conclude, the in vitro technique of gut sac nutrient incubation failed to support the assumption of nutrient directly stimulating IR-GLP-1(7-36)amide release from the small intestine.
Chapter 7.0
OVERALL CONCLUSIONS
As reviewed in Section 1.6, short term dietary change can influence GIP secretion in both man and animals. GIP and GLP-1(7-36)amide are considered currently to be the most potent incretin candidates. A wide study of the influence of hyperphagic conditions on the secretion of these two hormones was therefore undertaken in order to gain a better understanding of the relationship between the stimulus for and the secretion of these two hormones. In this thesis a range of physiological and pathological states have been investigated; all were believed to be characterised by a change in dietary consumption. In the case of the BB rats this was shown to be a false assumption, but the information obtained in the study of a spontaneous diabetic state was of value when compared with the information obtained with the chemically induced (streptozotocin) diabetic state. To review, the physiological and pathological states investigated were:

1. Pregnancy, a physiological condition of increased food consumption in rats in order to meet the nutritional demands of the growing foetus.

2. Lactation, a physiological condition of markedly increased food consumption as milk must meet all the nutritional requirements of the suckling neonates.

3. Foetus to neonate transition, a physiological time of sudden changes in nutrient supply. The timing of the supply changes from continuous to intermittent, and the nature of the supply also changes abruptly. In addition, the increasing nutritional requirements for growth have to be met. The ontogeny of GIP and GLP-1(7-36)amide at this time were investigated as were the influences of over and undernutrition during the suckling period. The importance of the maternal nutrient supply to the hyperinsulinaemia of the foetus during late gestation was also evaluated.
4. Cold acclimation, a physiological condition where food consumption was increased to meet the energy requirements of non-shivering thermogenesis.

5. Insulinoma, a short-term pathological condition that eventually culminates in severe hypoglycaemia. An increased food consumption was essential to counteract the hyperinsulinaemia and delay the neuroglycopenia.

6. Streptozotocin-induced diabetes, a pathological chemically induced insulin deficient condition where an increased food consumption is a characteristic of the hypoinsulinaemic-hyperglycaemic condition.

7. Spontaneous insulin-dependent diabetes in the BB rat, a pathological condition where insulin therapy is essential to survival.

8. Obese hyperglycaemic (ob/ob) mice, a pathological condition characterised by hyperinsulinaemia, hyperglycaemia, obesity and an increased food consumption.

In most of these studies a degree of hyperphagia was observed. Hyperphagia was defined as a sustained, significant increase in food consumption. The underlying cause of the hyperphagia was not investigated. The control of energy balance is thought to be regulated by a large number of neural, metabolic and endocrine factors (Williams & Bloom, 1989; Williams et al., 1991; Williams, 1990). The hypothalamus is involved in the regulation of energy balance and three nutritional balance regulating areas have been identified; the ventromedial nucleus, the paraventricular nucleus and the lateral hypothalamic area. However, other hypothalamic regions also influence appetite. Several neuropeptides which have been located within (but not exclusively) the hypothalamus have been identified (Williams et al., 1991). Interaction of these peptides with insulin status in the control of feeding behaviour and energy expenditure.
has been suggested (Williams et al., 1991). The insulin status of the animals studied in this thesis ranged from the excessive levels seen in the insulinoma-bearing animals to the deficient levels seen in the streptozotocin diabetic animals. The potential hypothalamic changes in the animals studied may therefore be very important in regulating the degree of hyperphagia observed which varied from a maximum 1.5-fold increase in food consumption in pregnant rats to a 3.6-fold increase in lactating rats.

Can a relationship between hyperphagia and IR-GIP concentration and IR-GLP-1(7-36)amide concentration be confirmed?

7.1 Hyperphagia and IR-GIP

When considering the plasma concentrations of this hormone, with the exception of the pregnant animals, an increased basal plasma IR-GIP concentration was seen in all hyperphagic animals. Where an 18 hour fast was imposed prior to acute tests, this significantly reduced the elevated plasma IR-GIP level towards normal (control) fasting values, confirming that hyperphagia is in part responsible for the increase in plasma IR-GIP concentration. The absence of a change in plasma IR-GIP concentration in the pregnant animals may well be due to the slow development of the hyperphagia in these animals. In the physiological studies investigated, the influence of hyperphagia on the acute response of IR-GIP to nutrient stimulation was not grossly modified during pregnancy or lactation and was only significantly modified in overnourished neonatal rats and after two weeks cold acclimation (but only to glucose stimulation) returning to normal after five weeks cold acclimation. During neonatal overnutrition, the acute IR-GIP response to an oral glucose tolerance test was significantly increased but was normalised three weeks after weaning. In the pathological states of insulinoma and streptozotocin, an increased plasma IR-GIP response to acute nutrient stimulation was again observed. The insulinoma animals
showed a heightened IR-GIP response to a corn oil suspension test and the streptozotocin animals showed a heightened response to a glucose test.

A relationship between hyperphagia and intestinal IR-GIP content was also present in both physiological and pathological states. Overnutrition during the suckling period revealed that after weaning, plasma and intestinal GIP levels were normalised, indicating no long-term effect of hyperphagia on entero-endocrine function. In some studies IR-GIP content of the small intestine was increased, in other studies only the concentration of IR-GIP was altered, and in others both parameters were affected by hyperphagia. The insulinoma-bearing animals were the only study population that did not show a significant increase in intestinal GIP content or concentration. However, these animals were only hyperphagic for six days and so the increased stimulation of the K cells was only short term, the very high levels of GIP secretion seen may well have limited cellular GIP storage. In addition, these animals had very high circulating insulin levels. The lack of effect of hyperphagia on intestinal IR-GIP may therefore be due to the rapid progression of these animals to a severe metabolic state.

It would appear that hyperphagia causes increased stimulation of the entero-endocrine K cell, resulting in increased IR-GIP secretion. In the majority of the physiological studies, hyperphagia did not overstimulate the K cell as there was no heightened acute IR-GIP response to a specific nutrient load in the pregnant, lactating and five weeks' cold-acclimated animals. The exceptions to this were the two-week cold-acclimated and the overnourished suckling rats. Other workers have suggested rapid stimulatory effects of increased food consumption on IR-GIP secretion (Section 1.6). This may be so, as in the studies reported here, basal plasma IR-GIP levels increased rapidly following the onset of the hyperphagia in all but the pregnant animals. However the influence of hyperphagia in physiological conditions appears not to be permanent. Reversal of the hyperphagia restored normal IR-GIP concentrations in the small intestine and plasma of overnourished suckling rats three weeks after weaning.
In the hyperphagic pathological states of insulinoma and streptozotocin diabetes, the acute IR-GIP response to specific nutrient loads was significantly increased. Interpretation of these findings is complicated by the altered insulin status of these animals. The influence of insulin status on IR-GIP secretion will be discussed later in this Chapter (Section 7.3).

7.2 Hyperphagia and IR-GLP-1(7-36)amide

A relationship between hyperphagia and intestinal IR-GLP-1(7-36)amide is not so clear. An increased IR-GLP-1(7-36)amide content and/or concentration of the small intestine was observed in the hyperphagic pregnant, lactating, cold acclimated, neonatally overnourished and streptozotocin diabetic rats studied. However the concentration of small intestinal IR-GLP-1(7-36)amide was not significantly modified during lactation in spite of the presence of a severe hyperphagia. In addition, the undernourished suckling neonatal rats also demonstrated a persistent increase in IR-GLP-1(7-36)amide content and concentration in their small intestines. Hyperphagia and semi-starvation therefore had a similar influence on the entero-endocrine L cell. The L cell in addition to secreting GLP-1(7-36)amide also secretes GLP-2, glicentin and oxyntomodulin. Glicentin and oxyntomodulin are often referred to by the old fashioned term enteroglucagon (Kreymann & Bloom, 1991). Enteroglucagon has been implicated in the control of intestinal growth (Section 3.3.4). The increase in IR-GLP-1(7-36)amide content of the intestine in undernourished and hyperphagic animals may therefore be the result of increased requirements for other hormones sequenced on the same proglucagon molecule. Certainly enteroglucagon itself may well be of importance during hyperphagia as increased wet weight of the small intestine was noted in all hyperphagic animals.

Chapter 6.0, the in vitro gut sac study, raised questions about the mechanism of stimulus-secretion coupling for GLP-1(7-36)amide. Simple sugars were shown to
directly stimulate IR-GIP release from the gut sac but not IR-GLP-1(7-36)amide release under the same conditions. The experimental procedure employed was very simple and straightforward and it is tentatively concluded that this hormone is not a true incretin hormone when assessed by the criteria listed in Table 1.1, Section 1.2.3. To support the theory that hyperphagia stimulates increased secretion of an entero-endocrine hormone would require the demonstration of nutrient stimulated secretion of the hormone. Glucose stimulation had no effect on GLP-1(7-36)amide secretion in vitro; further research in this area is required.

7.3 The Effect of Insulin on IR-GIP Secretion

The importance of insulin in the control of IR-GIP secretion was revealed by the contrasting pathological states of excessive and deficient insulin secretion, namely the insulinoma and streptozotocin diabetic studies. The insulinoma study revealed that in a hyperinsulinaemic environment fat stimulated acute IR-GIP secretion was maintained. This is believed to be due to insulin resistance, down regulation of the K-cell receptors for insulin. The streptozotocin study illustrated the contrasting situation of a loss of insulin feedback suppression on IR-GIP secretion and synthesis. Administration of exogenous insulin with a nutritional stimulus partially restored the feedback control on GIP secretion. It was clearly demonstrated that this feedback only acted in the presence of post-prandial nutrient levels in plasma. Both glucose and fat stimulated GIP secretion were seen to be suppressible by insulin.

7.4 The Role of GIP and GLP-1(7-36)amide in the EIA

Both GIP and GLP-1(7-36)amide were shown to be potent stimulators of insulin secretion in ob/ob and lean mice. From this it can be speculated that both are potent stimulators of the B-cell in rodents per se. GIP is a true incretin, being stimulated by the absorption of nutrients (Section 1.3.5). GLP-1(7-36)amide has also
been shown to be stimulated by nutrients (Section 1.4.6); however, doubt has been cast on whether the stimulus for secretion is direct nutrient stimulation of the L-cell. As discussed previously the importance of insulin feedback on GIP secretion was revealed by hypoinsulinaemic diabetes. In this situation increased plasma and intestinal GIP levels results from the hyperphagia and loss of insulin suppression. Long-term administration of insulin therapy to BB rats showed that intestinal GIP and GLP-1(7-36)amide concentrations can be normalised.

In the ontogeny study, a maintained increase in intestinal GIP and GLP-1(7-36)amide concentrations was observed in the first few days of extrauterine life. Insulin concentrations at the time were low yet normoglycaemia was preserved. The importance of direct actions of GIP and GLP-1(7-36)amide in adipose tissue metabolism at this time has been suggested. Additional support for this theory comes from the increased adiposity of rats overnourished during the suckling phase. The importance of this effect during cold acclimation was also suggested as a means of providing fuel for brown adipose tissue. Both GIP and GLP-1(7-36)amide were shown to have no effect on insulin secretion when administered under normoglycaemic conditions to ob/ob and lean mice. However, GIP secretion has been shown to be stimulated by oral fat (Kwasowski et al., 1986) and more recently GLP-1(7-36)amide has also been shown to be stimulated by oral fat alone in man (Elliott et al., 1991). The biological actions of these hormones at this time may therefore be on fat metabolism. The evidence for a role of these hormones in influencing the degree of adiposity is encouraging but is not complete (Sections 1.3.9 and 1.4.9). This proposed role for GIP and GLP-1(7-36)amide complements the incretin role of these peptides. They are thus capable of controlling plasma glucose post-prandially through augmentation of insulin secretion and enhancement of the effect of insulin on fatty acid synthesis from glucose. Independently GIP and GLP-1(7-36)amide have been shown to have effects on de novo fatty acid synthesis in vitro (Sections 1.3.9 and 1.4.9).
Increased nutrient absorption due to hyperphagia has been shown to stimulate increased GIP secretion. Fasting revealed the importance of the contribution of hyperphagia to the elevated levels of GIP seen in the studies reported here. Intestinal GLP-1(7-36)amide concentrations were not always elevated in hyperphagic animals, therefore the true nature of the stimulus for secretion is unknown. In the lactating rats insulin status was not modified. The rapid glucose disposal, due to milk production ensured that the increased plasma GIP levels did not augment insulin secretion.

The progressive hyperphagia of pregnancy and the physiological hyperinsulinaemia counteracted each other to maintain normal plasma GIP levels. In the ob/ob mice the hyperinsulinaemia is progressive and more severe. Changes in plasma GIP levels have been shown to mirror changes in plasma insulin. Both GIP and GLP-1(7-36)amide were potent stimulators of insulin secretion and would contribute to the hyperinsulinaemia. Together with insulin, GIP and GLP-1 contribute to adipose tissue lipogenesis. In ob/ob mice as the hyperinsulinaemia worsens, insulin resistance ensues. Insulin hypersecretion initially feeds back on GIP but as hyperinsulinaemia proceeds resistance causes reduced feedback on the K cell, therefore perpetuating the cycle of increased food intake, causing increased stimulation of the entero-insular axis, and thus promoting increased insulin secretion. Prolonged stimulation of adipose tissue by entero-endocrine hormones and insulin leads to excess fat deposition i.e. obesity. The importance of these hormones on adipose tissue metabolism is an important area of future research and offers an additional mechanism by which components of the entero-insular axis may contribute to obesity and non-insulin dependent diabetes syndromes.
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