# An investigation of secretion and metabolic effects of gastric inhibitory polypeptide in the ruminant.

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by

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## SUMMARY

The studies presented in this thesis were designed to evaluate the secretion and metabolic actions of GIP in ruminants. The initial study determined whether GIP is responsive to glucose and fat absorption in pre-ruminant and young ruminant goat kids (Experiment 3.1). Goats were surgically prepared to facilitate intraduodenal administration of nutrients and portal blood sampling. It was clearly demonstrated in both pre-ruminant and young ruminant goats that, as in simple-stomached animals, fat is a potent GIP secretagogue. It appeared, however, that ruminants and non-ruminants differ regarding the ability of glucose to elicit GIP secretion. Glucose absorption had no effect on GIP secretion in the pre-ruminant or ruminant animal.

Because GIP secretion may have been influenced by anaesthesia or surgical intervention it was decided to further investigate the GIP response to glucose, and to other nutrients, under more physiologically-normal conditions. In the pre-ruminant calf GIP secretion occurred within one hour of their normal milk feed (Experiment 3.2.a.). Postprandial GIP concentrations were also measured in pre-ruminant goat kids after ingestion of milk and milk constituents (Experiment 3.2.b). This study supported the observation made in anaesthetized goat kids that glucose absorption does not elicit GIP secretion, and that fat is a potent GIP secretagogue. The timing of the GIP response was also comparable to that observed in anaesthetized goat kids after intraduodenal injection of fat. Further studies in newborn goats (Experiment 3.2.c) demonstrated that suckling colostrum for the first time, immediately after birth, induced a GIP response of similar magnitude to that observed in older pre-ruminant goat kids after milk ingestion. Postprandial GIP concentrations were also determined in ruminant goat kids after ingestion of milk, milk constituents and cereal-based concentrates (Experiment 3.3). This study suggested that fat, and possibly protein, may induce GIP secretion in the ruminant.

Plasma GIP concentrations were shown to increase postprandially

after sheep ingested concentrates and hay (Experiment 4.1). The GIP response was delayed compared with the response to milk
in pre-ruminant and ruminant goat kids, but about the same (approximately 2 hours after feeding) as that after concentrates in the ruminant kids. Postprandial plasma GIP concentrations in the adult animals appeared to be directly related to the level of dietary intake, as indicated by studies investigating GIP secretion during the development of obesity in sheep (Experiment 4.2). Comparisons were made between lactating and non-lactating sheep (Experiment 4.3). Basal GIP levels and the GIP response to feeding were increased during lactation. This appeared to reflect an increase in dietary intake.

The metabolic effects of GIP on insulin secretion and adipose tissue metabolism were also evaluated in sheep. When GIP was intravenously injected with glucose, an insulinotrophic effect of GIP was not demonstrated (Experiment 5.1). However, this was consistent with the failure of glucose absorption to elicit GIP secretion in either sheep or goats. It seems unlikely that GIP is an incretin in ruminant species.

When the biological activity of different GIP preparations were tested by measuring their lipogenic effect in rat adipose tissue, all were confirmed to be biologically active (Experiment 5.2.a). Further studies in ovine adipose tissue demonstrated a moderate lipogenic effect of GIP compared with that of insulin (Experiment 5.2.b). These observations are consistent with a role for GIP in lipid metabolism.

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# CHAPTER 1

## Introduction

# INTRODUCTION

In simple-stomached animals, gastric inhibitory polypeptide (GIP) is released from the small intestine in response to nutrient absorption. Once secreted into the circulation, GIP is known to act in at least two ways in the regulation of nutrient utilization. First, GIP is an important component of the enteroinsular axis and augments insulin secretion in response to nutrient absorption. Secondly, GIP exerts direct anabolic effects in adipose tissue.

Relatively little is known of the secretion and actions of gut hormones in ruminants, where they could have important implications for lactation and growth. A large, and increasing number of gut hormones have been identified, some of which are also found in the brain. The major ones are shown in Table 1.1. GIP-secreting cells are present in ruminants, but differences in digestive processes between ruminants and simple-stomached animals could alter the regulation of GIP secretion. Metabolic differences, notably the reliance on gluconeogenesis for glucose supply, could modify actions of GIP in ruminants. An understanding of the secretion and actions of GIP in ruminants may ultimately allow identification of dietary and/or immunological approaches designed to improve efficiency of utilization of dietary nutrients for production, and to manipulate carcass or milk composition.

In Chapter 1 of this thesis the literature relating to the secretion and actions of GIP in simple-stomached animals, and to digestion and metabolism in ruminants, is reviewed as an introduction to a series of investigations of the regulation of GIP secretion and of possible actions of the hormone in ruminant species.

## 1.1. Structure and cellular localisation of GIP

GIP is well established as a gut hormone with physiological

**Table 1.1.** Distribution and functional categories of gut peptides (after Morgan, Oben, Marks and Fletcher, 1992).

| Peptide | Distribution | | | Functional Category |
|---|---|---|---|---|
| | Brain | Gastro-Intestinal Tract | | |
| | | Neural | Endocrine | |
| Secretin | | | + | Endocrine |
| Glucagon peptides | | | + | Endocrine |
| GIP | | | + | Endocrine |
| Motilin | | | + | Endocrine/ Paracrine |
| Gastrin | +? | | + | Endocrine |
| Pancreatic polypeptide | + | | + | Endocrine/ Neurocrine |
| Cholecystokinin | + | + | + | Endocrine/ Neurocrine |
| Somatostatin | + | + | + | Endocrine/ Paracrine/ Neurocrine |
| Opioid peptides | + | + | | Neurocrine |
| Tachykinins | + | + | | Neurocrine |
| Vasoactive intestinal peptide | + | + | | Neurocrine |

actions in simple-stomached animals. GIP was purified and the amino acid sequence determined (Jornvall, Carlquist, Kwauk, Otte, McIntosh, Brown and Mutt, 1981) after Brown and Pederson (1970) had isolated a component from a partially-purified cholecystokinin-pancreozymin (CCK) preparation, extracted from porcine small intestine, that exhibited gastric acid inhibitory properties other than those attributable to CCK (Brown, Mutt and Pederson, 1970).

Porcine GIP was originally thought to contain 43 amino acids (Brown and Dryburgh, 1971), but was subsequently found to consist of 42 amino acid residues (Jornvall *et al.*, 1981). Even the purest GIP preparations contained a minor peptide component with a sequence almost identical to that of GIP (1-42). This truncated component, GIP (3-42), lacks the first two amino acids, tyrosine and alanine (Jornvall *et al.*, 1981). Human GIP has been isolated from postmortem intestinal tissue. There are small differences in the amino acid sequence compared with that of porcine GIP, with the arginine at position 18 in porcine GIP replaced with histidine, and the serine at position 34 by asparagine (Moody, Thim and Valverde, 1984). The peptide sequence of bovine GIP differs from porcine GIP by the substitution of an isoleucine residue for lysine at position 37 (Carlquist, Maletti, Jornvall and Mutt, 1984). Different forms of immunoreactive GIP, with molecular weights of 5000 and 8000 Daltons, have been measured in blood and tissue extracts after chromatographic purification (Brown, Dryburgh, Ross and Dupre, 1975; Dryburgh, 1977; Jorde, Amland, Burhol, Giersky and Ebert, 1983). The level of the 8 KDa form is only moderately elevated postprandially and has no apparent insulinotrophic effect (Krarup, 1988).

GIP-containing cells have been identified in the small intestine of a variety of species by radioimmunoassay of tissue extracts and by immuno-fluorescence techniques (Polak, Bloom, Kuzio, Brown and Pearse, 1973; Bloom 1974; Gaginella, Mekhjian and O'Dorisio, 1978; Buchan, Ingman-Baker, Levy and Brown, 1982; Usellini, Capella,

Solcia, Buchan and Brown, 1984). The cell type that contains GIP has been designated the K cell (Solcia, Capella, Vasallo and Buffa, 1974). The intestinal localization of immunoreactive-GIP in humans (Bloom and Polak, 1978) is shown in Figure 1.1, with highest GIP concentrations located in the duodenum. Patterns of distribution are similar in dogs (O'Dorisio, Cataland, Stevenson and Mazzaferri, 1976) and pigs (Ponter, Salter, Morgan and Flatt, 1991). In the pre-ruminant calf, goat kid, and in the adult sheep (Figure 1.2) GIP-immunoreactivity is limited to the small intestine and absent from the reticulum, rumen, omasum, abomasum and large intestine (Bunnett and Harrison, 1986).

The ontogenic development of GIP-containing cells in the human gastrointestinal tract has been investigated, with GIP cells detected in the proximal duodenum of the foetus by week 14 of the gestation period and subsequently in the distal small intestine (Bryant, Buchan, Gregor, Ghatei, Polak and Bloom, 1982; Leduque, Gespach, Brown, Rosselin and Dubois, 1982).

## 1.2. Regulation of GIP secretion in simple-stomached animals

### Effects of carbohydrate on GIP secretion

The ability of oral glucose to stimulate GIP secretion in simple-stomached animals has long been established (Kuzio, Dryburgh, Malloy and Brown, 1974; Cataland, Crockett, Brown and Mazzaferri, 1974). Monosaccharides must be actively transported across the brush border membrane of the enterocyte by the $Na^+$/glucose cotransporter to cause GIP secretion. Thus, absorption of fructose and mannose, which does not involve this transport system, fails to induce GIP release (Sykes, Morgan, English and Marks, 1980; Creutzfeldt, Ebert, Nauck and Stockmann, 1983), and ouabain, an inhibitor of $Na^+/K^+$-ATPase, (Ebert and Creutzfeldt, 1983) and phlorizin, a competitive inhibitor of the $Na^+$/glucose cotransporter, curtail glucose-stimulated GIP secretion (Creutzfeldt and Ebert, 1977; Sykes *et al.*, 1980). GIP responses to

**Figure 1.1.** The distribution of cells containing GIP immunoreactivity in human intestine as described by Bloom and Polak (1978).

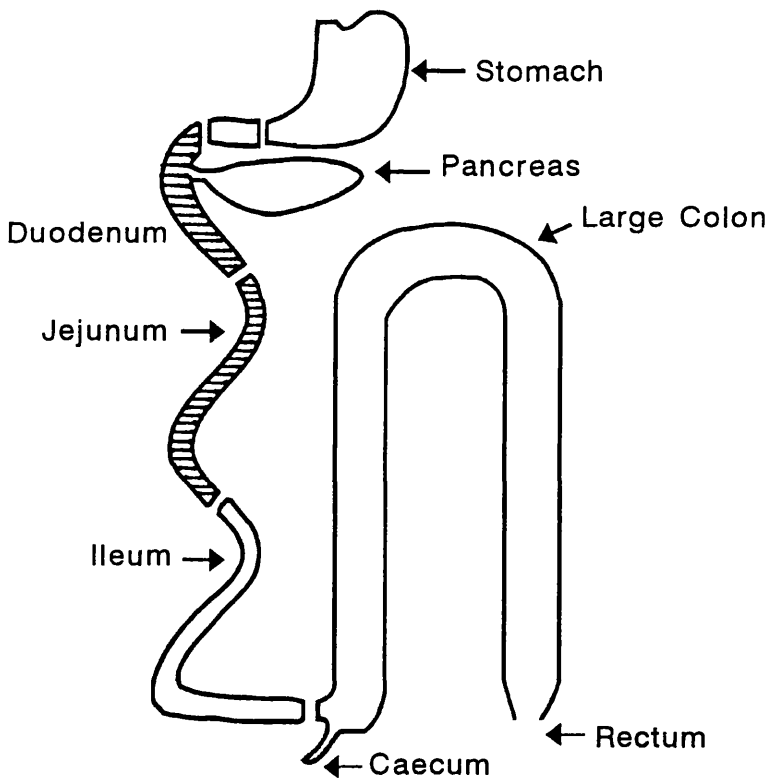
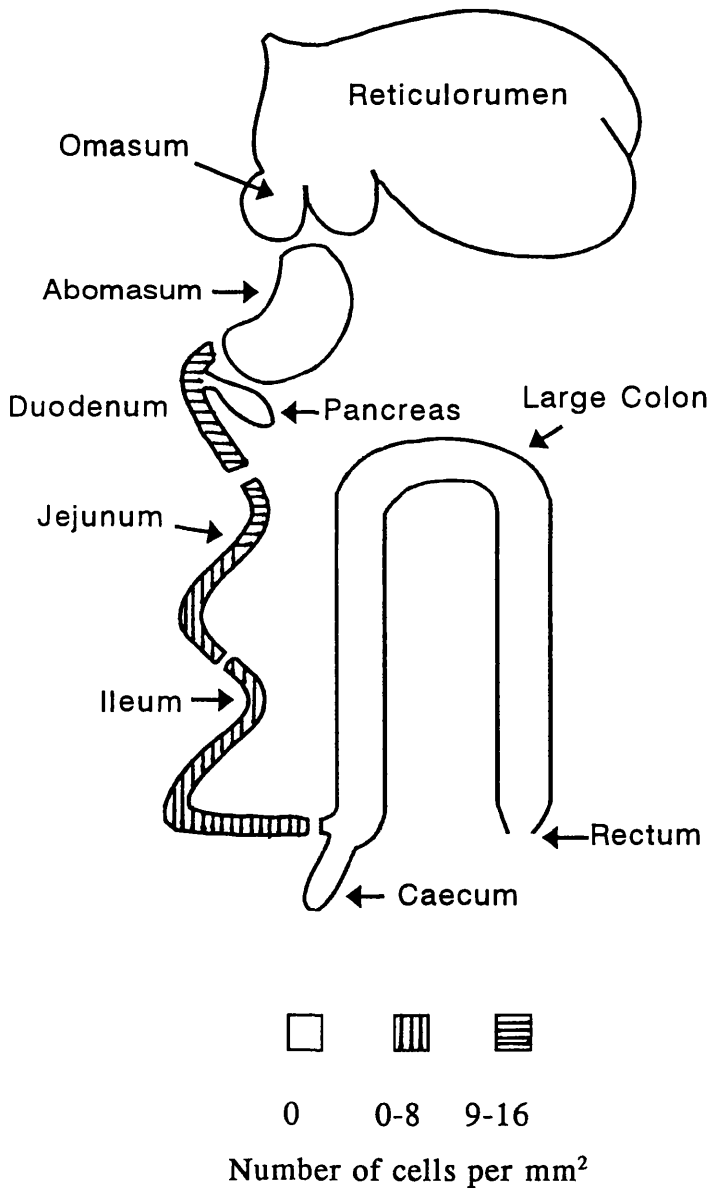


**Figure 1.2.** The distribution of cells containing GIP immunoreactivity in sheep intestine as described by Bunnett and Harrison (1986).



glucose analogues demonstrate that the response to actively-absorbed monosaccharides is not dependent upon their subsequent metabolism or passage, via a facilitated glucose transporter, across the basolateral membrane of the enterocyte into blood; 3-O-methylglucose, which is not metabolised within intestinal cells, and $\alpha$-methylglucoside, which is not a substrate for the transporter at the basolateral membrane, elicit GIP release (Sykes *et al.*, 1980; Flatt, Kwasowski and Bailey, 1989). However, the demonstration in ob/ob mice, that fructose and 2 deoxy-glucose can increase plasma GIP concentration indicate that, in some circumstances, the $Na^+$/glucose cotransporter may not be the only mechanism whereby carbohydrate evokes GIP secretion (Flatt, Kwasowski, Bailey and Bailey, 1989).

GIP concentrations in portal blood increase significantly within 1 minute of duodenal administration of glucose in anaesthetized rats (Schulz, Burhol, Jorde and Waldum, 1981), with the rise in GIP concentration increasing as increasing amounts of glucose were given. Martin, Sirinek, Crockett, O'Dorisio, Mazzaferri, Thompson and Cataland (1975) showed a similar dose-dependency for glucose-induced GIP secretion in dogs and, since no GIP response was observed to a hyperosmolar solution of mannitol, suggested that duodenal osmoreceptors are not involved in stimulation of GIP release. Increasing GIP responses following graded oral doses of glucose have also been reported in man (Schlesser, Ebert and Creutzfeldt, 1986).

Polysaccharides and disaccharides stimulate GIP release only if broken down to actively-absorbed monosaccharides. In humans, the GIP response after sucrose ingestion is delayed relative to that after glucose, and this has been attributed to the time taken by intestinal brushborder enzymes to hydrolyse the sucrose to fructose and glucose before the absorption of the individual monosaccharides (Creutzfeldt, Ebert, Caspary, Folsch and Lembcke, 1979). Acarbose, an $\alpha$-glucoside hydrolase inhibitor, abolishes GIP secretion in response to an oral sucrose load (Folsch, Ebert and Creutzfeldt, 1981).

**Effect of fat on GIP secretion**

Fat is a potent GIP secretagogue (Brown, 1974; Falko, Crockett, Cataland and Mazzaferri, 1975), and GIP secretion in response to triacylglycerol ingestion is dependent upon fatty acid absorption. Thus, cholestyramine, which impairs micelle formation and decreases fat absorption, leads to reduced GIP secretion (Ebert and Creutzfeldt, 1983). Similarly, GIP secretion in patients with chronic pancreatitis, and associated fat malabsorption, is increased by the addition of digestive pancreatic enzymes to the meal (Ebert and Creutzfeldt, 1980).

Fatty acids differ in their ability to induce GIP secretion. In a study by Kwasowski, Flatt, Bailey and Marks (1985) with fasted obese hyperglycaemic (ob/ob) mice, intraduodenally-administered saturated short-chain (propionic C3:0) and saturated medium-chain fatty acids (capric C10:0), failed to elicit GIP secretion. However, both saturated (stearic acid, C18:0) and unsaturated (oleic C18:1, linoleic C18:2, linolenic C18:3 acids) long-chain fatty acids gave rise to significant GIP responses. Similarly, studies in man have shown that intraduodenal infusion of long-chain but not of medium-chain fatty acids enhances GIP secretion (Ross and Shaffer, 1981).

The length of the carbon chain of fatty acids determines the pathway of intestinal absorption. Short- and medium- chain fatty acids are transferred across the intestinal cells without esterification and enter the portal vein as free fatty acids, whereas long-chain fatty acids are esterified before incorporation into chylomicrons and secretion into the general circulation via the lymphatic system (Clement, 1980). Thus, esterification within the enterocyte, an energy-dependent process, may be a prerequisite for GIP secretion. Consistent with this it has been reported that the hydrophobic surfactant Pluronic L-81 inhibits GIP release during long-chain fatty acid absorption by blocking chylomicron formation (Tso, Balint and Rodgers, 1981; Ebert and Creutzfeldt, 1984). The degree of saturation of long-chain fatty acids affects their melting point and fluidity within the intestinal lumen; this may influence

their rate of absorption and explain the apparently more potent GIP-releasing effect of unsaturated long-chain fatty acids compared with saturated long-chain fatty acids (Kwasowski *et al.*, 1985).

**Effect of protein on GIP secretion**

It was first shown by Thomas, Mazzaferri, Crockett, Mekhjian, Gruemer and Cataland (1976) that intraduodenal administration of amino acids could stimulate GIP secretion, although to a lesser degree than fat and carbohydrate. Subsequent studies revealed specificity in the nature of the response. A mixture consisting of arginine, histidine, isoleucine, lysine and threonine caused greater GIP release than a mixture containing methionine, phenylalanine, tryptophan and valine (Thomas, Sinar, Mazzaferri, Cataland, Mekhjian, Caldwell and Fromkes, 1978). Flatt, Kwasowski, Howland and Bailey (1991), studying the effects of oral administration of individual amino acids in ob/ob mice, found no differences between the GIP responses to alanine, arginine, cysteine, histidine, hydroxyproline and lysine, but that glycine and threonine did not elicit GIP secretion. Thus, there was no indication of a link between specific transport mechanisms for amino acids (Munck, 1981) and GIP secretion from the K cell. Other research groups have shown that protein ingestion does not elicit GIP secretion when given as a meat extract (Brown, 1974), fillet steak (Cleator and Gourlay, 1975) or turkey steak (Elliot, Morgan, Tredger, Deacon, Wright and Marks (1993). This could be related to insufficient breakdown of protein to individual amino acids prior to absorption.

**Modification of GIP responses**

In line with the relationship between the amount of nutrient absorbed and GIP secretion, and with the concentration of K cells in the duodenum and jejunum, dietary manipulations that alter the rate, or site, of nutrient absorption can modify GIP secretion. Furthermore, dietary changes can influence the concentrations of GIP in intestinal tissue, and

this too may alter the GIP response to a standard meal.

Diets enriched with sucrose have been shown to increase GIP secretion in response to oral sucrose (Reiser, Michaelis, Cataland, and O'Dorisio, 1980). This could be attributable to enzyme induction, resulting in a more rapid hydrolysis of sucrose into monosaccharides. By contrast, carbohydrate meals supplemented with the gel-forming soluble fibre, guar gum, exhibit a decreased rate of glucose absorption from the small intestine and a diminished GIP response (Blackburn, Redfern, Jarjis, Holgate, Hanning, Scarpello, Johnson and Read, 1984).

The effect of a high fat diet upon enhancing GIP secretion is more pronounced than that observed with high carbohydrate diets. Studies have shown rats to be responsive to variations in dietary fat content, with enhanced GIP and insulin secretion observed after a high-fat diet (Hampton, Kwasowski, Tan, Morgan and Marks, 1983), perhaps relating to an increase in releasable GIP in intestinal tissues associated with a higher energy intake (Ponter *et al.*, 1991). A high-fat cafeteria-style diet in rats has been shown to increase GIP and insulin responses to oral glucose compared with animals fed on standard laboratory chow (Tan, Kwasowski and Marks, 1987). In contrast, a low-fat diet in humans attenuates the GIP response to an oral fat load (Morgan, Tredger, Hampton, French, Peake and Marks, 1988); this could be caused by a decreased rate of fat absorption. The factors involved in the dietary manipulation of GIP secretion are complex, and care must be taken when extrapolating between experiments, especially where these have been conducted in different species. This appears to be particularly so where the customary diet differs in composition; as discussed by Morgan (1992), rats appear to be especially sensitive to changes in the fat content of the diet, which is normally low, providing less than 10 % of energy intake, as compared with man, where approximately 40 % of dietary energy is in the form of fat. It is interesting to note that in this respect diets for ruminants resemble those for rats, in that fat typically constitutes 3 to 5 % of the total ration.

However, as will be discussed later, because of intervention of microbial activity in the rumen the pattern of nutrients digested in the small intestine is closer to that in man as much of the digestible carbohydrate is fermented and the volatile fatty acids (VFA) produced are absorbed from the rumen.

There appears to be hormonal, as well as dietary, modification of GIP secretion. A negative feedback loop has been proposed between GIP and insulin. It has been shown that fat-induced secretion of GIP can be attenuated during intravenous administration of glucose (Cleator and Gourlay, 1975; Crockett, Cataland, Falko and Mazzaferri, 1976; Ross and Dupre, 1978; Ebert, Frerichs and Creutzfeldt, 1979; Verdonk, Rizza, Nelson, Go, Gerich and Service, 1980) or exogenous insulin (Brown *et al.*, 1975). A direct effect of insulin on K cells has been demonstrated (Stockmann, Ebert, Creutzfeldt, 1984; Takahashi, Manaka, Katsuyuki, Fukase, Tominaga, Sasaki, Kawai and Ohashi, 1991), though where both blood glucose and insulin are elevated, part of the limitation to GIP secretion is attributable to a decreased rate of gastric emptying (Morgan, Hampton, French, Peake and Marks, 1988). Glucose-stimulated GIP secretion is not under the same control mechanism and is not reduced by endogenous (Anderson, Elahi, Brown, Tobin and Andres, 1978) or exogenous insulin (Elahi, Anderson, Debas, Hershcope, Raizes, Tobin and Andres, 1979; Creutzfeldt, Talaulicar, Ebert and Willms, 1980). Feedback of insulin on fat-stimulated GIP secretion after the consumption of a mixed meal perhaps modulates GIP release during the earlier stages of digestion when both glucose and fat are being absorbed and when insulin secretion is sufficient to effect disposal of triacylglycerol. Subsequently, as the rate of glucose absorption and consequently insulin secretion decline the GIP response to fat absorption is restored when, as described later, GIP will not enhance insulin secretion but will assume a greater importance in triacylglycerol disposal. Additionally, loss of feedback as the result of the preceding period of hyperinsulinaemia (Stockmann *et al.*, 1984) may

limit the extent to which fat-induced GIP secretion is depressed. The loss of feedback inhibition of insulin on fat-induced GIP release in obesity has been suggested by Hampton *et al.* (1983) to be caused by a decreased response of K cells to insulin because of a reduction in the numbers of insulin receptors.

It has been suggested that over-secretion of GIP is a factor in the development of obesity. However, evidence for this is equivocal, and any exaggeration of GIP responses appears to relate in part to effects (as discussed above) of a period of a high level of feed intake on rate of gastric emptying, efficiency of intestinal digestion and/ or amount of releasable GIP in intestinal tissues. GIP responses in obese individuals revert to normal after 5 days of food restriction (Willms, Ebert and Creutzfeldt, 1978). Also, Ebert and Creutzfeldt (1989) found GIP responses to an intraduodenally-administered test meal to be similar in obese and lean subjects. The feedback control of insulin on fat-stimulated GIP may also be impaired in obese subjects, though this too appears to be restored after a period of food restriction (Ebert *et al.*, 1979). Thus, hyperGIPaemia associated with diet-induced obesity seems to be largely a consequence of the consumption of high levels of an energy-rich diet. As will be discussed later, the metabolic effects of the elevated GIP contribute to the hyperinsulinaemia and the accumulation of adipose tissue in these circumstances. The effects on GIP secretion appear to be accentuated in genetically-obese animals, where hyperplasia of K cells, high basal concentrations of GIP, exaggerated GIP responses to nutrients and greater sensitivity to dietary changes have been observed (see Morgan, 1992).

### 1.3. Actions of GIP in simple-stomached animals

As well as inhibiting gastric acid secretion, GIP (though generally only when administered in supraphysiological amounts) inhibits gastrin and pepsin secretion and gastrointestinal motility, increases mesenteric blood flow, alters the electrolyte composition of saliva, and although not

detected in the brain, influences the release of follicle-stimulating hormone and growth hormone (See Brown, Buchan, McIntosh and Pederson, 1989). However, the major effects of GIP relate to its insulinotrophic action and to direct, insulin-like, effects on lipid metabolism. High- and low-affinity binding sites for GIP have been detected on insulinoma plasma membranes (See Brown *et al.*, 1989; Malletti, Portha, Carlquist, Kergoat, Laburthe, Marie and Rosselin, 1984), and receptors have been reported on stomach, small and large intestine, and various muscle groups (Whitcomb, O'Dorisio, Nishikawara, Shetzline and Cataland, 1983). However, the presence of receptors on adipose tissue has yet to be demonstrated, but this has been attributed to the existence of a relatively small number of high-affinity sites or to damage during cell or membrane isolation (Brown *et al.*, 1989).

**Effect of GIP on insulin secretion**

Oral glucose is more effective in stimulating insulin release than glucose administered intravenously in amounts sufficient to give comparable levels of glycaemia. This led to the suggestion that oral glucose gave rise to the secretion from the intestine of an insulinotrophic factor (Elrick, Stimmler, Hlad and Arai, 1964; McIntyre, Holdsworth and Turner, 1964). The term entero-insular axis was introduced by Unger and Eisentraut (1969) to describe all the stimuli from the small intestine that contribute to insulin secretion postprandially, including hormonal, neuronal and direct substrate stimulation. The relative importance of these components has been estimated; in response to a liquid test meal in rats, the neural component accounted for 20 %, and hormonal factors for 30 % of the stimulation of insulin secretion (Berthoud, 1984).

GIP is considered to be a major hormonal component of the entero-insular axis (Creutzfeldt and Ebert, 1985), potentiating insulin secretion under physiological conditions. The insulinotrophic effect of

GIP is dependent on glucose concentration and there exists a glucose concentration threshold of 5.5 mM in man, approximately 1.4 mM above basal, below which GIP will not stimulate insulin release (Elahi *et al.*, 1979). Thus, fat-stimulated GIP secretion is not associated with insulin secretion unless hyperglycaemia is achieved, for example after ingestion of a mixed meal or by intravenous glucose infusion (Cleator and Gourlay, 1975; Crockett *et al.*, 1976). GIP released in response to amino acid absorption can increase amino acid-stimulated insulin release independently of blood glucose concentration (Mazzaferri, Ciofalo, Waters, Starich, Groshong and De Palma, 1983) such that, as seen for glucose, insulin responses to amino acid mixtures are greater after intraduodenal rather than intravenous administration (Thomas *et al.*, 1976).

The insulinotrophic action of GIP occurs in a dose-dependent manner (Pederson and Brown, 1976), and there has been some debate as to whether GIP is insulinotrophic at physiological concentrations. Sarson, Wood, Kansal and Bloom (1984) reported that in man exogenous administration of porcine GIP to achieve concentrations comparable to those observed after oral glucose was not insulinotrophic unless the level of glycaemia was supraphysiological. This has been attributed (Brown *et al.*, 1989; Marks, Morgan, Oben and Elliot, 1991) to differences in the avidity for human and porcine GIP of the antiserum used to measure endogenous GIP. Other workers have shown physiological concentrations of exogenous GIP to have a strong insulinotrophic action (Nauck, Bartels, Orskov, Ebert and Creutzfeldt, 1973; Dupre, Ross, Watson and Brown, 1973). Results of a recent study by Nauck, Bartels, Orskov, Ebert and Creutzfeldt (1993) using infusions of synthetic human GIP indicate that GIP makes a major contribution to the incretin effect after oral glucose.

GIP has been reported to account for approximately 50 % of the difference in insulin secretion between an oral versus intravenous glucose load, as determined by studies involving immuno-neutralization

(Ebert and Creutzfeldt, 1982; Ebert *et al.*, 1983). Gut-derived factors other than GIP are known to stimulate insulin secretion. It has been shown that certain glucagon-like peptides (GLP) are insulinotrophic in the hyperglycaemic state. GLP-1 (7-36) amide, recently discovered in the terminal ileum and colon, and the subject of many recent reviews (for example, Fehmann, Goke, and Goke, 1992; Orskov, 1992) may prove to be at least as important as GIP in potentiating nutrient-stimulated insulin secretion. On a molar basis, GLP-1 (7-36) amide is a more potent stimulator of insulin secretion than GIP (Shima, Hirota and Ohboshi, 1988) but increases in circulating levels of GLP-1 are smaller than those in GIP in response to oral glucose or a test meal (Kreymann, Williams, Ghatei and Bloom, 1987; Takahashi *et al.*, 1990). Studies indicate additive effects of GLP-1 (7-36) amide and GIP; both GLP-1 (7-36) amide and GIP mediate insulin secretion by adenylate cyclase (Goke, Trautmann, Haus, Richter, Fehmann, Arnold and Goke, 1989), which could explain the additive effect of the hormones at submaximal effective concentrations (Fehmann, Goke, Goke, Trautmann and Arnold, 1989).

The combination of GIP and CCK has been shown to enhance glucose-induced insulin secretion in both perifused islets (Zawalich, 1988) and in mice *in vivo* (Ahren and Lundquist, 1983). Because the insulinotrophic effect of CCK is mediated via the phospholipase C-mediated hydrolysis of membrane phosphoinositides, potentiation of the effects of GIP and GLP-1 by CCK can be explained via the interaction of different second messenger systems (Zawalich, 1988; Fehmann, Goke, Weber, Goke, Trautmann and Arnold, 1990).

It has been suggested that the insulinotrophic effect of GIP can be modulated by neural factors (McCullough, Marshall, Bingham, Rice, Manning and Kalhan, 1985), but recent studies have shown the insulin-releasing effect of GIP to be unaffected by transplantation of the pancreas (Clark, Wheatley, Brons, Bloom and Calne, 1989).

**Effect of GIP on adipose tissue metabolism**

In addition to effects on the pancreas, GIP has direct actions in other tissues, mainly relating to various aspects of adipose tissue metabolism: *de novo* synthesis of fatty acids, uptake of preformed fatty acid from triacylglycerol of plasma lipoproteins, and lipolysis. The overall effect of these actions is to promote fat deposition.

Physiological concentrations of porcine GIP (0.2 to 4 ng/ml) have been shown, by measuring the incorporation of radiolabelled acetate into fatty acids in rat adipose tissue, to stimulate fatty acid synthesis (Oben, Morgan, Fletcher and Marks, 1991). Supraphysiological concentrations of GIP (5 to 500 ng/ml) also enhance the uptake and incorporation of glucose into extractable lipid in rat adipocytes (Hauner, Glotting, Kaminska and Pfeifer, 1988). Direct insulin-like effects with GIP have been shown in ovine adipose tissue perfusates *in vivo* where reduced concentrations of glucose in the perfusate during intravenous GIP infusion indicated stimulation of lipogenesis (Martin, Faulkner and Thompson, 1993). Furthermore, Haji Baba and Buttery (1991) reported a strong positive effect of GIP on acetate incorporation in ovine adipose tissue *in vitro*.

In studies with 3T3-L1 cells, a mouse embryo fibroblast cell line resembling adipocytes, GIP stimulates lipoprotein lipase (LPL), the enzyme which hydrolyses the triacylglycerol component of circulating lipoprotein particles prior to fatty acid uptake by tissues. Physiological levels of GIP increased LPL secretion into the culture medium and enhanced enzyme activity in acetone-ether extracts of the adipocytes (Eckel, Fujimote and Brunzell, 1978). More recently, Knapper, Puddicombe, Morgan, Fletcher and Marks (1993) demonstrated that GIP stimulates LPL activity in rat adipose explants. The effect of exogenous GIP on the clearance of chylomicrons from blood has been investigated with chyle from donor dogs fitted with thoracic duct catheters. GIP enhanced the removal of chylomicron triacylglycerol, indicating a role in the clearance of lipids postprandially (Wasada, McCorkle, Harris,

Kawai, Howard and Unger, 1981). However, the elimination rate of a fat emulsion (Intralipid) infused intravenously in man in the post prandial state, after fasting, and during intravenous infusion of GIP failed to show an effect of either endogenous or exogenous GIP (Jorde, Petterson and Burhol, 1984). Similarly, a study in dogs failed to show GIP-enhanced removal of triacylglycerol (Intralipos) after an oral glucose or galactose load (Ohneda, Kobayashi and Nikei, 1983). However, increased chylomicronaemia after treatment with antibodies to GIP, in rats consuming fat, is consistent with the involvement of GIP in adipose tissue metabolism (Kwąsowski, Tan, De Silva and Marks, 1984).

As the consumption of glucose is known to enhance the affinity of the insulin receptor (Muggeo, Bar and Roth, 1977) and adipose tissue responsiveness to insulin, it has been postulated that GIP may play a role in this increased cellular sensitivity (Livingston and Moxley, 1982). Indeed, there is evidence that some of the direct effects of GIP on adipose tissue are, in part, insulin-dependent. Studies using adipocytes from epididymal fat pads of Sprague-Dawley rats have shown that GIP enhances both insulin receptor affinity and insulin-stimulated glucose uptake (Starich, Bar and Mazzaferri, 1985). Beck and Max (1983) demonstrated that GIP could enhance insulin-stimulated fatty acid ($^{3}$H-palmitate) incorporation into rat adipose tissue. Further studies, using the same experimental system, demonstrated differences in sensitivity to GIP in epididymal fat pads of Zucker (fa/fa) rats and their lean littermates (fa/-). It was suggested that adipose tissue in the obese Zucker (fa/fa) rat was hypersensitive to the action of GIP and this sensitivity may play a role in the development of obesity by promoting efficient utilization of ingested fat (Beck and Max, 1987).

The amino acid sequence of GIP resembles those of glucagon, secretin, and VIP (Dupre, Greenidge, McDonald, Ross and Rubinstein, 1976). The possibility that there are interactions between these peptides has been studied. GIP is lipolytic, but the effect is weak compared with

that of glucagon and GIP has been shown *in vitro* to inhibit lipolysis stimulated by glucagon but not that stimulated by secretin or VIP in rat adipocytes (Dupre *et al.*, 1976). Further studies in adipocytes demonstrated GIP was capable of selectively blocking glucagon activation of adenylate cyclase, possibly by displacing glucagon from its receptor (Ebert and Brown, 1976).

## 1.4. Aspects of digestion and metabolism in ruminants

### The ruminant digestive tract

Ruminants develop several pouches anterior to the region corresponding to the gastric stomach of simple-stomached animals. These pouches are the rumen, reticulum and omasum. Of these, the rumen and reticulum are separated only by a fold in the stomach wall and are functionally related, and often described as the reticulo-rumen. The reticulo-rumen is the largest compartment and is the region of the gastrointestinal tract were a microbial population exists in a symbiotic relationship with the host animal. The microbes ferment dietary material, thereby providing the ATP, carbon dioxide and ammonia required for microbial growth and forming, as waste-products VFA, which are absorbed by the host. Dietary components, bacteria and bacterial waste-products not absorbed from the reticulo-rumen flow through the omasum, where electrolytes and water are removed, to the abomasum. This is the true stomach, so called because it corresponds in function to the fundic and pyloric regions of the non-ruminant stomach. It is in the abomasum that the digesta are first subjected to the digestive processes of the host. From the abomasum digesta flow into the duodenum, where bile and pancreatic enzymes breakdown bacteria and undegraded food residues to sugars, long-chain fatty acids and amino acids, prior to absorption. Undigested material passes from the small intestine to the caecum and colon, where further microbial fermentation and some absorption of VFA occurs.

**Processes of digestion**

Carbohydrates typically comprise 70-90 % of the dry matter in diets for ruminants. This carbohydrate is present as simple sugars and storage polysaccharides such as starch in plant cell contents, and as the structural polysaccharides cellulose, hemicellulose and pectins in plant cell walls (Morrison, 1979). Dietary sugars are almost completely fermented in the rumen (Beever, Thompson and Harrison, 1971). Little of the starch present in the food normally escapes microbial fermentation; for a typical barley-based diet the $\alpha$-glucoside entering the small intestine, including that of microbial origin, is equivalent to less than 10 % of that ingested, though this can be as much as 30 % when a slowly fermented starch such as maize is given (Armstrong and Smithard, 1979). Pectins are readily fermented, but the extent of cellulose and hemicellulose breakdown is dependent on the level of feeding and the degree of lignification of the cell walls, and generally about 50 % of that in the diet is digested by rumen microorganisms (Mitchell, Little, Karr and Hayes, 1967; Watson, Savage, Brown, and Armstrong, 1972). The initial product of starch and cellulose breakdown in the rumen is glucose, which undergoes glycolysis. The pyruvate formed is present in the rumen in very low concentrations as it is rapidly metabolised to VFA, $CO_2$ and methane. The major VFA are acetate, propionate and n-butyrate with small amounts of n-valerate, isovalerate and isobutyrate. These are absorbed across the rumen wall.

Although glucose does not normally reach the small intestine, post-ruminally administered glucose is absorbed (Kreikemeier, Harmon, Brandt, Avery and Johnson, 1991). The capacity for active absorption of glucose during short-term infusion appears to be limited (Kreikemeier *et al.*, 1991), but the $Na^+$/glucose transporter, which falls to negligible amounts after weaning, has been shown to be induced in the small intestine of adult sheep during a 4-day period of duodenal infusion of glucose (Shirazi-Beechey, Hirayama, Wang, Scott, Smith and Wright, 1991).

Starch reaching the small intestine is subjected to the actions of pancreatic and intestinal α-amylase, maltase and isomaltose. There is still some debate as to whether enzymatic capacity limits intestinal starch digestion, but in general this is thought to be unlikely for typical diets for ruminants (Owens, Zinn and Kim, 1986), and some degree of adaption in amyloyltic and disaccharase activities to dietary changes have been observed (Harmon, 1992). Any starch escaping digestion is likely to be fermented by the increasing number of microorganisms in the distal small intestine (Mayes and Orskov, 1974). Substantial amounts of glucose have been shown to appear in mesenteric blood in sheep given a maize-based diet (Janes, Parker, Weekes and Armstrong, 1984). However, studies in cattle by Huntington and Reynolds (1986) showing that only 65 and as little as 8 % respectively of abomasally infused glucose and starch was recovered as net portal glucose absorption and by Kreikemeier *et al.* (1991) where about 35 % of starch disappearing from the small intestine appeared as net portal glucose absorption have led to uncertainties concerning the extent to which carbohydrate disappearing from the small intestine is fermented by microorganisms therein or is metabolised by the gut tissue.

Post-ruminal digestion of hemicellulose and cellulose, being the result of microbial fermentation, is largely confined to the caecum and colon. VFA are absorbed from this region, but the remaining fermentation products are lost with the food residues in the faeces.

Lipids comprise less than 5 % of the dry matter in diets typically fed to ruminants. The lipids undergo rapid and complete hydrolysis as the result of microbial activity in the rumen (Garton, Lough and Vioque, 1961). The glycerol produced is rapidly fermented to VFA, principally propionate (Hobson and Mann, 1961). There is negligible degradation of long-chain fatty acids within the rumen (Garton, 1969) but unsaturated C18 fatty acids are extensively hydrogenated by the rumen bacteria (Bickerstaffe, Noakes and Annison, 1972). The amount of fatty acids flowing to the duodenum exceeds dietary intake reflecting a

contribution from *de novo* synthesis by rumen microorganisms (Knight, Sutton, Storry and Brumby, 1978). Except where dietary fat has been protected from breakdown in the rumen, for example by coating with formaldehyde-treated protein, lipid entering the duodenum consists mainly of unesterified saturated fatty acids, predominantly 18:0, adsorbed onto particulate matter (Scott, Ulyatt, Kay and Czerkawski, 1969). The fatty acids are solubilised by the action of bile and pancreatic juice, and are efficiently absorbed, even when fatty acid intake is greatly increased (Heath and Hill, 1969). In sheep given normal diets, about 20 % of the fatty acids absorbed from the small intestine disappeared from the upper jejunum, where the pH of the digesta was 3.6-4.2, and about 60 % was absorbed from the middle and lower jejunum, where the pH was 4.7-7.6; fatty acid absorption was virtually complete at the ileum (Lennox and Garton, 1968). Although extensive hydrolysis of lipid occurs in the rumen, there is significant lipase activity in the pancreatic secretion of ruminants; though this is lower than that of non-ruminants triacylglycerol is digested and absorbed efficiently when, for example, protected fat supplying up to 1.5 kg fatty acids/day is given to dairy cows (Storry, Brumby and Dunkley, 1980).

Absorption is thought to be a passive process, dependent on the maintenance of an inward diffusion gradient by the binding of the fatty acids to intracellular proteins and the re-esterification of absorbed fatty acids. As described by Brindley (1984), in line with the preponderance of unesterified fatty acids in the lipid absorbed by ruminants, under normal circumstances triacylglycerol are resynthesised in the enterocyte via the $\alpha$-glycerophosphate pathway but the monoacylglycerol pathway, which predominates in simple-stomached animals absorbing considerable amounts of 2-monoacylglycerol, assumes greater importance when protected fats are given. In terms of the forms of lipoprotein in which absorbed lipid is exported to the lymph, whilst in simple-stomached animals triacylglycerol is preferentially incorporated into chylomicrons,

and less into very-low-density lipoproteins (VLDL), in the ruminant triacylglycerol is preferentially incorporated into VLDL. This is thought (see Moore and Christie, 1984) to reflect the relatively slow, steady rate of fat absorption in ruminants, allowing the synthesis of surface film components of the lipoprotein to keep pace with triacylglycerol synthesis, and the saturated nature of the absorbed fatty acids since this appears to favour the formation of VLDL rather than chylomicrons.

Dietary protein is hydrolysed in the rumen by the microorganisms to yield peptides, amino acids and ammonia. Non-protein nitrogen also contributes amino acids and ammonia, and urea re-entering the rumen from blood and saliva further adds to ammonia production (see Orskov, 1982). Feedstuffs differ widely in the ruminal degradability of their protein; for most diets about 60 % of dietary protein is degraded (Satter and Roffler, 1977). The non-protein nitrogen compounds in the rumen are used for microbial protein synthesis, at least 70 % of which is derived from ammonia. The efficiency of this depends on the extent to which the availability of ammonia and energy are matched. When insufficient energy is available rumen ammonia is not captured (Satter and Slyter, 1974) and diffuses across the rumen wall and passes to the liver, where it is converted to urea.

In lactating cows 50-60 % of the total protein entering the duodenum is likely to be of bacterial origin (Hagemeister, Kaufmann and Pfeffer, 1976), the amino acids composition of which varies little with diet (Weller, 1957). The digestion of protein is initiated in the highly acidic conditions of the abomasum by the peptic enzymes of the abomasal secretions. Because of the relatively low concentrations of bicarbonate in ruminant pancreatic juice a low pH extends further along the small intestine than in simple-stomached animals (Ben Ghedalia, Tagari, Bondi and Tadmor, 1974) and this may limit proteolytic activity in the early small intestine, but apart from this difference the processes of digestion and absorption of proteins appear to be similar to those in simple-stomached animals (Webb and Bergman, 1991). The digestibility

of undegraded dietary protein varies widely with source, but in the region of 60-70 % of microbial protein is digested in the small intestine, with a further 10-20 % fermented in the large intestine where the uptake of nitrogen is mostly in the form of ammonia (Ulyatt, Dellow, Reid and Bauchop, 1975).

As shown in Table 1.2, intervention of the ruminal micro-organisms in the digestive process has important implications for the pattern of products of digestion absorbed in ruminants.

**Table 1.2.** Estimated absorption of products of digestion by the lactating dairy cow for a range of mixed forage and concentrate diets (from Thomas and Rook, 1983).

| Product of Digestion | Gross Energy Absorbed (MJ/d) | Weight (kg/d) |
|---|---|---|
| Short-chain fatty acids | | |
| Total | 117 - 147 | |
| Acetic acid | 43 - 75 | 2.9 - 5.1 |
| Propionic acid | 31 - 57 | 1.5 - 2.7 |
| Butyric acid | 24 - 37 | 1.1 - 1.5 |
| Long-chain fatty acids | 19 - 54 | 0.5 - 1.4 |
| Amino acids | 30 - 45 | 1.3 - 1.9 |
| Glucose | 4 - 17 | 0.3 - 1.0 |

**Glucose metabolism**

Although, under normal dietary conditions, and particularly in animals receiving high forage diets, only small amounts of glucose are absorbed directly from the ruminant digestive tract, on a metabolic liveweight basis, glucose utilization rates show glucose to be quantitatively almost as important in ruminants as in non-ruminants (Annison and White, 1961; Ballard, Hanson and Kronfield, 1969). Glucose is essential for ruminant brain and erythrocyte metabolism (Lindsay, 1980) and as a precursor for muscle glycogen, and it is also utilized for the generation of NADPH required for lipogenesis.

Normally, 90 - 100 % of the glucose supply to ruminant tissues is derived by gluconeogenesis (Lindsay, 1978). The precursors include propionate and amino acids absorbed from the digestive tract, glycerol from triacylglycerol breakdown, lactate from brain, erythrocyte and muscle glycolysis and amino acids from protein turnover. Acetate is not gluconeogenic but is an alternative substrate to glucose for oxidation in skeletal and cardiac muscle, adipose tissue, liver, kidney and the lactating mammary gland and for lipogenesis (Annison and Linzell, 1964, Holdsworth, Neville, Nader, Jarret and Filsell, 1964; Bird, Chandler and Bell, 1981).

Approximately 85 % of gluconeogenesis occurs in the liver (Bergman, Katz and Kaufman, 1970) and the remainder in the kidney (Kaufman and Bergman, 1971). Propionate, the only major ruminal VFA capable of contributing to glucose synthesis (Bergman, 1973), may contribute as much as 40 % of the total glucose produced in the fed animal. The other glucogenic VFAs, isobutyric and valeric acid contribute about 5 % (Lindsay, 1978). The second important exogenous source of glucogenic substrate is amino acids absorbed from the small intestine. Many of the major amino acids, lysine, leucine and tryptophan being notable exceptions, can contribute to glucose synthesis via pyruvate or TCA cycle intermediates (Thomas and Rook, 1983). Most of the amino acids in the portal blood are removed by the liver, the

uptake of some exceeding the amount absorbed from the small intestine. As the rate of triacylglycerol turnover in adipose tissue is normally slow, little glycerol is released to become available for gluconeogenesis. Approximately 50 % of that removed by the liver and kidneys is utilised for glucose synthesis, contributing about 5 % of the total glucose supply in the fed animal (Bergman, Starr and Reulein, 1968).

Glucose requirements are greatly increased during lactation to meet the requirements for lactose synthesis (Bickerstaffe, Annison and Linzell, 1974). It has been calculated that a cow producing 40 kg of milk requires more than 3 kg glucose/day (Young, 1977). There is a two- to three-fold increase in gluconeogenesis (Bergman and Hogue, 1967); food intake and hence supply of glucogenic precursors is increased, as are the activities of the major glucogenic enzymes, pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose diphosphatase and glucose 6-phosphatase (see Vernon, 1988). Reduced insulin secretion during lactation (Lomax, Baird, Mallinson and Symonds, 1979) encourages the release of glucogenic precursors from peripheral tissues and, furthermore, Bassett (1978) has suggested that the reduced insulin:glucagon ratio has a role in promoting gluconeogenesis. In addition, although bovine mammary cells have insulin receptors (Oscar, Baumrucker and Etherton, 1986) mammary glucose transport does not appear to be sensitive to insulin (Laarveld, Christensen and Brockman, 1981), thus the lower insulin concentrations favour glucose uptake by the gland.

**Lipid metabolism**

In ruminants, the liver is of minor importance in lipogenesis and adipose tissue is the main site for the uptake, synthesis and storage of lipids (Ballard *et al.*, 1969). Ruminants differ from simple-stomached animals in that acetate is the major source of carbon for fatty acid synthesis with lesser contributions from other precursors including glucose, amino acids, lactate and propionate (Vernon, Finley and

Taylor, 1985). Butyrate and β-hydroxybutyrate, which is formed during absorption of butyrate from the rumen, can as CoA esters replace acetyl CoA as primers for fatty acid synthesis in the ruminant mammary gland (Moore and Christie, 1979) and probably in adipose tissue also (Bauman, 1976). NADPH required for fatty acid synthesis in adipose tissue is generated by the metabolism of glucose in the pentose phosphate pathway (Vernon, 1980) and glucose and acetate in the isocitrate dehydrogenase cycle (Bauman, 1976). Glucose is also the main precursor of glycerol-3-phosphate required for fatty acid esterification (Vernon, 1980). As in other species, exogenous fatty acids derived from the triacylglycerol of plasma lipoproteins by the action of LPL are taken up and re-esterified in ruminant adipose tissue (Vernon, 1980).

Lipolysis involves the hydrolytic cleavage of triacylglycerol by a hormone-sensitive lipase (HSL) to free fatty acids (FFA) and glycerol. The rate of release of these fatty acids from the adipose tissue depends not only on the rate of triacylglycerol hydrolysis but also on the rate at which fatty acids are re-esterified within the tissue (Vernon and Flint, 1983).

Increases in food intake in early lactation do not keep pace with demands of milk synthesis, and animals are normally in negative digestive energy balance and mobilising considerable amounts of adipose tissue at this time. The rate of fatty acid synthesis falls in ovine adipose tissue during early lactation (Vernon, Clegg and Flint, 1981), as does the activity of the regulatory enzyme in this process, acetyl Co A carboxylase (Vernon, Faulkner, Finley, Pollock and Taylor, 1987). Rates of triacylglycerol synthesis show the same pattern, and are paralleled by changes in activity of two key enzymes of esterification, glycerol 3-phosphate acyltransferase and glycerol 3-phosphate dehydrogenase (Vernon *et al.*, 1987). The availability of preformed fatty acid for esterification is also lower as LPL activity decreases in adipose tissue in ruminants during early lactation (Shirley, Emery, Convey and Oxender, 1973; Vernon, Clegg and Flint, 1981). Reductions in lipid

synthesis are accompanied by increased lipolysis in adipose tissue; the active form of HSL is increased (Sidhu and Emery, 1972) and this, together with the decreased activities of the enzymes of triacylglycerol synthesis, promotes the release of FFA, especially since glucose fails to stimulate re-esterification of fatty acids in early lactation (Metz and van den Bergh, 1977). There is a concurrent increase in mammary gland LPL activity (Shirley *et al.*, 1973). As insulin is the major anabolic hormone, promoting lipid synthesis and inhibiting lipolysis in adipose tissue, the fall in insulin concentration contributes to these adaptations during lactation. In addition, though the number and affinity of insulin receptors on ovine adipose tissue are unchanged during lactation, the sensitivity and responsiveness of the tissue appears to be diminished, presumably reflecting modification of the intracellular process which occur after the hormone has bound to its receptor (Vernon and Taylor, 1988).

## 1.5 Aims and objectives

The aims of the experiments presented in this thesis were 1) to determine whether GIP is responsive to nutrient absorption in ruminant species and to identify the specific nutrients that elicit GIP secretion, 2) to investigate whether GIP is insulinotrophic in ruminants, and 3) to evaluate the possible lipogenic effect of GIP in ruminant adipose tissue.

Studies focused principally on sheep and goats for practical reasons. However, it was hoped to make use of the comparative aspects of using two closely related species, and of data from studies conducted where possible in calves, to gain an indication of the situation in the dairy cow, this being an animal of considerable commercial value and importance.

### 1) Nutrients involved in GIP secretion

In view of the differences in patterns of digestion and nutrient absorption between simple-stomached animals and ruminants, one of the first objectives was to determine whether nutrient-induced GIP secretion occurs in ruminants.

Because digestion in young pre-ruminants resembles that in simple-stomached animals, initial studies were conducted in pre-ruminant, as well as ruminant, goat kids. In a preliminary attempt to confirm GIP secretion glucose and fat, selected because they are potent GIP secretagogues in simple-stomached animals, were administered intraduodenally and GIP concentrations were measured in portal blood. This approach necessitated the use of general anaesthesia and considerable surgical intervention. Subsequently, therefore, GIP responses in the general circulation were to be measured in similar animals, fitted with jugular catheters, given meals of milk and individual milk constituents, the aim being to assess the ability of specific nutrients to evoke GIP secretion under physiological conditions. Any differences between pre-ruminants and young ruminants may be indicative of GIP responses which, although not normally expressed in the adult ruminant,

might be important in the neonate and might also be restored in the adult under certain dietary conditions.

In terms of the adult ruminant, studies of GIP secretion were to be conducted in adult sheep to determine whether circulating GIP concentrations respond to consumption of conventional feedstuffs, *i.e.* cereal-based concentrates and hay, and whether responses are modified in animals in different physiological states, *i.e.* obesity and lactation, associated with increased levels of dietary intake.

**2) Effects of GIP on insulin secretion**

Given the peculiarities of glucose metabolism in the ruminant, effects of GIP on insulin secretion, which enhance glucose disposal in simple-stomached animals, may be modified. To investigate whether the insulinotrophic effect of GIP is retained in ruminants the ability of intravenously-administered GIP to enhance the insulin response to an intravenous glucose load was to be determined in adult sheep.

**3) Effect of GIP on adipose tissue**

Direct metabolic effects of GIP on glucose metabolism in adipose tissue of simple-stomached animals have been described. Studies were therefore conducted to determine whether such effects also exist in ruminant adipose tissue, where acetate rather than glucose is the main lipogenic precursor. Initially, the lipogenic effect of GIP in rat adipose tissue was to be reproduced, as a means of evaluating the biological activity of a variety of GIP preparations, before assessing the effect of the most potent on acetate incorporation into ruminant adipose tissue. The activity of LPL in rat adipose tissue is enhanced by GIP, which suggests that GIP released during the absorption of fat may be involved in nutrient utilization. It was also intended to investigate whether GIP enhances LPL activity in ruminant adipose tissue.

# CHAPTER 2

## Materials and methods

## 2.1 PREPARATION OF RADIOLABELLED GIP

The iodination procedure used during this study was based on the method described by Morgan, Morris and Marks (1978), in which $^{125}$Iodine was incorporated into GIP using the chloramine-T method (Greenwood, Hunter and Glover, 1963). Synthetic porcine GIP (Peninsula, St Helens, U.K.) was used for the preparation of the radiolabel. GIP was dissolved in 0.4 M phosphate buffer, pH 7.5, batched in 5 $\mu$g (10$\mu$l) aliquots in eppendorf tubes and stored at -20°C.

The iodination was performed by adding 500 $\mu$Ci (5 $\mu$l) $^{125}$I-sodium iodide (IMS. 30, Amersham International, Amersham, U.K.) to a 5 $\mu$g aliquot of GIP. The reaction was started by the addition of chloramine-T (15 $\mu$g in 10 $\mu$l 0.4 M phosphate buffer, pH 7.4) and was stopped after 15 seconds by the addition of sodium metabisulphate (40 $\mu$g in 20 $\mu$l phosphate buffer, pH 7.4). Reaction products were diluted by the addition of 200 $\mu$l phosphate buffer (0.4 M, pH 7.4, containing 0.5 % Bovine Serum Albumin (BSA)) and transferred to a 1 × 15 cm column (Biorad, Hemel Hempstead, U.K.) of Sephadex G15 (Sigma, Poole, U.K.), which had previously been equilibrated with acetate buffer (0.1 M, pH 5.0, containing 0.5 % BSA and 500 Kallikrein inhibitory units (KIU)/ml aprotinin (Sigma, Poole, U.K.). Each fraction eluted with acetate buffer from the column was collected for 2 minutes and its level of radioactivity determined using a Geiger counter (Mini-instruments Ltd, Essex, U.K.) at a distance of 40 cm. Iodinated GIP eluted in the first peak and free iodine in the second peak (Figure 2.1). The two GIP fractions with the highest level of radioactivity were combined, then diluted with 1 ml acetate buffer. The radiolabelled GIP was aliquoted (100 $\mu$l), stored at -20°C, and was stable under these conditions for 1 month.

Incorporation of $^{125}$Iodine into GIP was confirmed by trichloroacetic acid (500 $\mu$l, 10 % w/v) precipitation of a 50 $\mu$l GIP fraction of defined radioactivity. After incubation for 10 minutes at

room temperature, the precipitate was recovered by centrifuging for 2 minutes in a benchtop centrifuge and the level of radioactivity in the precipitate determined. In a successful iodination more than 95 % of $^{125}$Iodine in the GIP fraction was precipitated.

The specific activity of $^{125}$I-GIP (typically 80 $\mu$Ci/$\mu$g) was calculated using the following equation:

$$\text{Specific activity } (\mu Ci/\mu g) = \frac{(B + D)}{(B + D + E)} \times \frac{A}{1000} \times \frac{500}{C}$$

A = $^{125}$I added to GIP (cps at 60 cm; where 500 $\mu$Ci = 1000 cps)

B = residual $^{125}$I not transferred to the column (cps at 60 cm; assumed to be $^{125}$I-GIP)

C = Amount of GIP iodinated ($\mu$g)

D = $^{125}$I present in GIP peak (cps at 40 cm)

E = $^{125}$I present in iodine peak (cps at 40 cm)

## 2.2 GIP RADIOIMMUNOASSAY

### Reagents

RIA buffer:

0.05M $Na_2PO_4$, pH 7.4

0.15 M NaCl

0.05 % (w/v) sodium azide

0.5 % (w/v) Bovine Serum albumin (BSA)

500 KIU aprotinin/ml (Sigma, Poole, U.K.)

GIP standard:

Natural porcine GIP (Guildhay Antisera Ltd, Guildford, U.K.) dissolved in RIA buffer, batched in 100 $\mu$l aliquots (40 ng/ml) in eppendorf tubes and stored at -20°C.

First antibody:

Rabbit anti-porcine GIP antiserum (Guildhay Antisera Ltd,

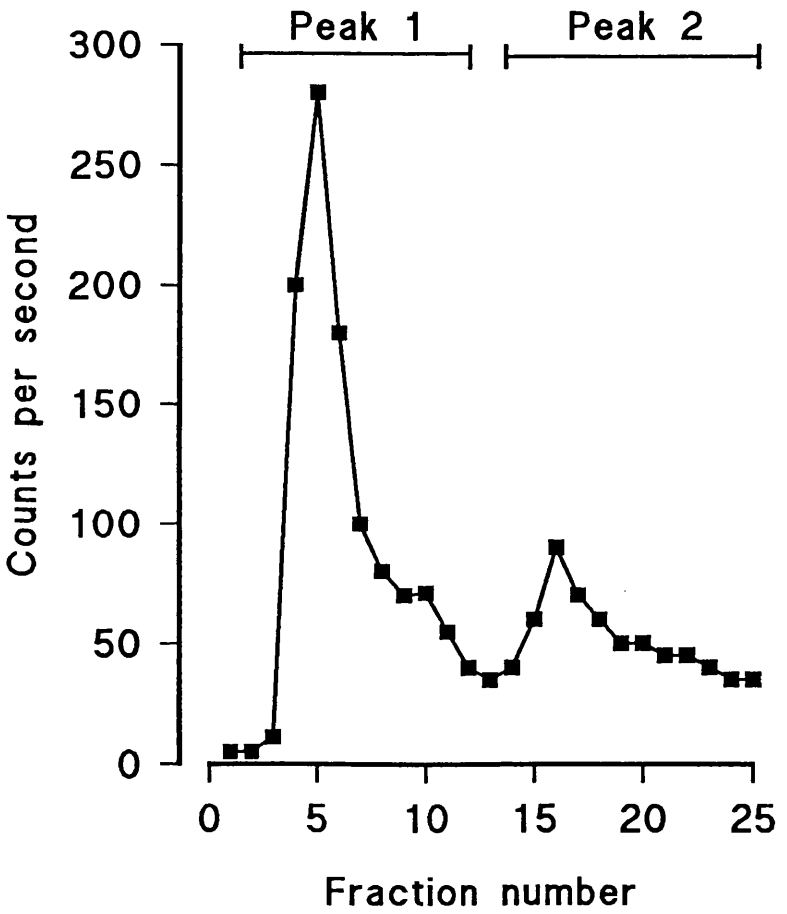



**Figure 2.1.** Separation of iodinated GIP from unbound iodine on a sephadex G15 column. $^{125}$I-GIP was eluted in the first peak and free iodine in the second peak. Fractions 4 to 6, containing precipitable $^{125}$I-GIP, were combined and used as radiolabel in the GIP radioimmunoassay.

Guildford, U.K.). Final dilution in the assay of 1:20000.

Second antibody:

140 mg ethylene diaminetetra-acetic acid (EDTA) in 15 ml RIA buffer (adjusted to pH 7.4 after adding EDTA), then 5 μl normal rabbit serum and 125 μl anti-rabbit precipitating serum (Scottish Antibody Production Unit, Glasgow, U.K.) and 15 ml of 16 % (w/v) polyethylene glycol (PEG).

**Assay procedure**

The GIP assay was based on the method described by Morgan, Morris and Marks (1978). GIP standards ranged from 0.125 ng/ml to 4 ng/ml. A volume of 50 μl was used for standards (quadruplicates) and samples (duplicates) and to this was added 100 μl of rabbit anti-porcine GIP antiserum. Buffer (50 μl) was added to give a total volume of 200 μl and final antiserum dilution of 1:20000. To the zero tubes was added 100 μl buffer and 100 μl antiserum, whereas the non-specific binding tubes received 200 μl buffer only. After a 48-hour incubation at 4°C, $^{125}$I-GIP was added to all tubes (50 μl: 10000 cpm). Tubes were incubated for 24 hours at 4°C, then 250 μl of second antibody were added. The tubes were incubated for 4 hours at room temperature, then centrifuged at 3000 rpm for 30 minutes and the supernatant decanted. The protein precipitate, containing antibody-bound $^{125}$I-GIP, was counted on a gamma counter (Cobra Auto-gamma; Packard, Pangbourne, Berks, U.K.). The concentration of GIP in the samples was determined by interpolation from the standard curve (Figure 2.2).

The sensitivity of the assay was defined as the lowest concentration of GIP distinguishable from zero. To determine this, the standard deviation of 20 zero tubes was determined and the sensitivity limit was taken as the point on the standard curve which corresponded to a decrease of two standard deviations from zero binding. The sensitivity limit was 0.25 ng/ml. The intraassay coefficient of variation was 8.7 %.

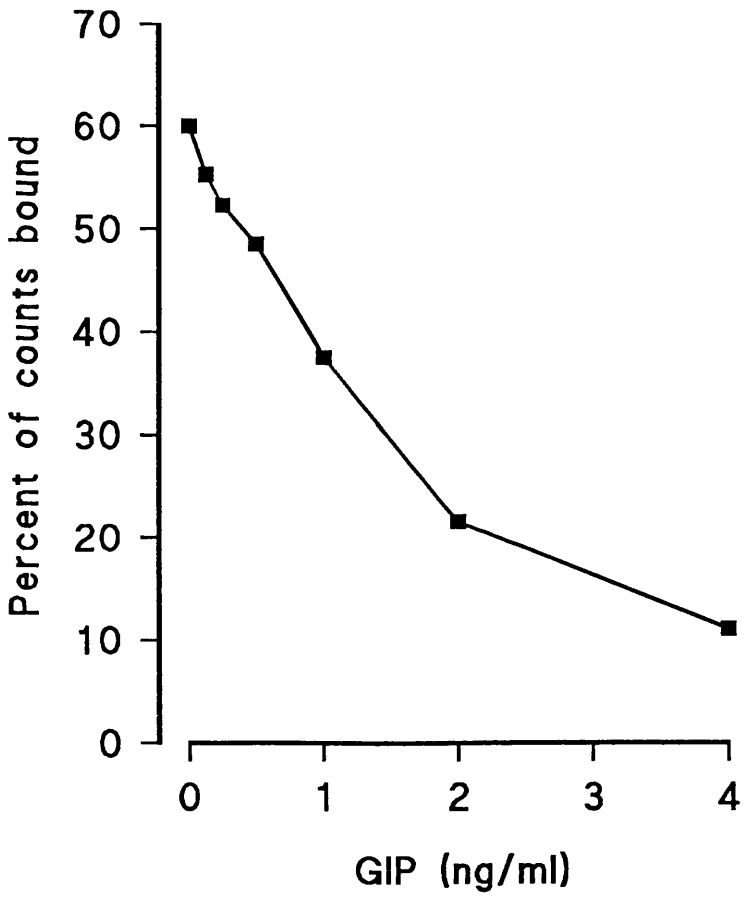



**Figure 2.2.** Standard curve obtained with GIP. Values were determined in quadruplicate. Data points represent mean value (S.E.M. too small to record).

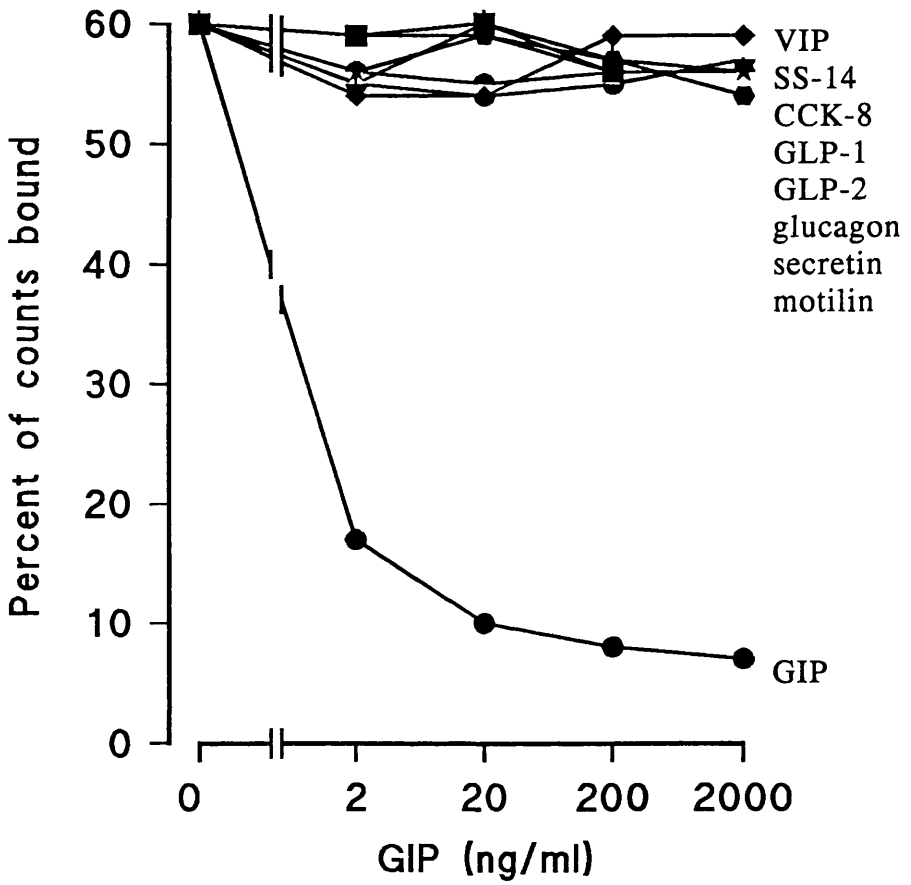



**Figure 2.3.** Cross-reactivity of rabbit anti-porcine GIP antiserum.

The antiserum showed no cross-reactivity with glucagon, GLP-1 (7-36)-$NH_2$, GLP-2, somatostatin-14 (SS-14), vasoactive intestinal peptide (VIP), secretin, motilin and CCK-8 when incubated with up to 2000 ng/ml of the various peptides (Figure 2.3).

## 2.3 INSULIN RADIOIMMUNOASSAY

### Iodination of insulin

The iodination procedure used was based on a method first described by Fraker and Speck (1978), in which $^{125}$Iodine was incorporated into protein using the Iodogen$^{Tm}$ reagent (Pierce Europe BV, Oud-Beijerland, Netherlands). Microtubes were coated with 30 $\mu$l of Iodogen reagent (50 $\mu$g/ml in chloroform) evaporated to dryness at room temperature and stored at -20°C. The iodination was performed by adding 500 $\mu$Ci (5 $\mu$l) $^{125}$I-sodium iodide (IMS. 30, Amersham International, Amersham, U.K.) to a 5 $\mu$g (5 $\mu$l) aliquot of insulin in an Iodogen-coated microtube with 10 $\mu$l phosphate buffer (0.4 M, pH 7.4). After 15 minutes, the reaction products were diluted with 200 $\mu$l of KI (2 % w/v) and separated on a column of Sephadex G 25 (Sigma, Poole, U.K.), which had previously been equilibrated with RIA buffer.

### Reagents

RIA buffer:

0.05M $Na_2PO_4$, pH 7.4

0.15 M NaCl

0.05 % (w/v) sodium azide

0.5 % (w/v) Bovine Serum Albumin (BSA)

Insulin standard:

Natural porcine insulin (Sigma, Poole, U.K.) dissolved in 10 mM HCl then diluted in RIA buffer, batched in 100 $\mu$l aliquots (10 $\mu$g/ml) in eppendorf tubes and stored at -20°C.

First antibody:

Anti-bovine insulin antiserum (raised in guinea pigs by D.J.Flint, Hannah Research Institute). Final dilution in the assay of 1:20000.

Second antibody:

140 mg ethylene diaminetetra-acetic acid (EDTA) in 15 ml RIA buffer (adjusted to pH 7.4 after adding EDTA), then 5 $\mu$l normal guinea pig serum and 125 $\mu$l anti-guinea pig precipitating serum (Scottish Antibody Production Unit, Glasgow, U.K.) and 15 ml of 16 % (w/v) polyethylene glycol (PEG).

**Assay procedure**

The insulin assay was based on a method described by Vernon, Clegg and Flint (1981). Insulin standards ranged from 0.16 to 5 ng/ml. A volume of 100 $\mu$l was taken, in duplicate, for standards and samples and to this was added 100 $\mu$l of anti-bovine insulin antiserum to give a final antiserum dilution of 1:20000. To the zero tubes was added 100 $\mu$l buffer and 100 $\mu$l antiserum, whereas non-specific binding tubes received only 200 $\mu$l buffer. After a 24 hour incubation at 4°C, $^{125}$I-insulin was added to all tubes (100 $\mu$l:10000 cpm). Tubes were incubated for 24 hours at 4°C, then 300 $\mu$l of second antibody added. Tubes were incubated for 4 hours at room temperature, then centrifuged at 3000 rpm for 30 minutes and the supernatant decanted. The protein precipitate, containing antibody-bound $^{125}$I-insulin, was counted on a gamma counter (Cobra Auto-gamma, Packard, Pangbourne, Berks, U.K.). The concentration of insulin in the samples was determined by interpolation from a standard curve.

## 2.4 PLASMA GLUCOSE ASSAY

Plasma glucose concentrations were determined using a method described by Bergmeyer and Bernt (1974). Glucose was oxidised by the

enzyme glucose oxidase to give hydrogen peroxide and glucuronic acid. The hydrogen peroxide then reacted with O-dianiside to yield a coloured product. The final colour intensity was proportional to the glucose concentration.

$$\text{1)}\quad \text{Glucose} + 2H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Glucuronic acid} + 2H_2O_2$$

$$\text{2)}\quad \underset{\text{(Colourless)}}{H_2O_2 + \text{O-Dianisidine}} \xrightarrow{\text{Peroxidase}} \underset{\text{(Brown)}}{\text{Oxidised O-Dianisidine}}$$

**Reagents**

Combined Enzyme-Colour Reagent

Sodium phosphate buffer, 0.5M, pH 7.0; 10ml

O-dianisidine (1 % w/v in 95 % ethanol); 50 $\mu$l

Peroxidase, 40 units/ml

Glucose oxidase, 30 units/ml

Glucose standard, 1mM

**Procedure**

The analysis was performed using a Titerteck analyser and 96-well plates. To the 7 wells nominated as standards was added 0, 5, 10, 15, 20, 25, and 30 $\mu$l glucose standard (1mM). Plasma sample (5 $\mu$l) was added to the remaining wells. Combined Enzyme-Colour Reagent Solution (250 $\mu$l) was added to all wells and mixed thoroughly. After an incubation period of 30 minutes at 37°C, the absorbance was read at 450 nm, using the well with no glucose or sample as a blank. The glucose concentration in the sample was determined by interpolation from the standard curve.

## 2.5 TRIACYLGLYCEROL ASSAY

Plasma triacylglycerol concentrations were determined using a

commercial kit (Sigma, Poole, U.K.), for the enzymatic hydrolysis of triacylglycerol to glycerol and fatty acids, followed by the enzymatic measurement of released glycerol.

$$\text{1) Triacylglycerol} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{2) Glycerol} + \text{ATP} \xrightarrow[\text{kinase}]{\text{Glycerol}} \text{Glycerol-1-phosphate} + \text{ADP}$$

$$\text{3) Phosphoenol pyruvate} + \text{ADP} \xrightarrow[\text{kinase}]{\text{Pyruvate}} \text{Pyruvate} + \text{ATP}$$

$$\text{4) Pyruvate} + \text{NADH} + \text{H}^{+} \xrightarrow[\text{dehydrogenase}]{\text{Lactate}} \text{Lactate} + \text{NAD}^{+}$$

NADH absorbs at 340 nm, thus the decrease in absorbance was directly proportional to the triacylglycerol concentration in the sample.

**Reagents**

Reagent A

ATP, 0.40 mM

NADH, 0.27 mM

Phosphoenol pyruvate, 0.5 mM

Buffer, pH 7.2

Lactate dehydrogenase, 3 units/ml

Lipase, 30 units/ml

Pyruvate kinase, 2 units/ml

Reagent B

Glycerol kinase, 16 units/ml

**Procedure**

The analysis was performed using a Titerteck analyser and 96-well plates. Distilled water (300 $\mu$l) was pipetted into the well

nominated as the blank. Distilled water (50 $\mu$l) was added to the well of the reagent blank. To the remaining wells was added 50 $\mu$l of plasma sample. Reagent A (250 $\mu$l) was added to the reagent blank and samples, then mixed thoroughly. The absorbance of the samples ($A_1$) and reagent blank ($B_1$) was read at 340 nm. Reagent B (10 $\mu$l) was added to the reagent blank and samples, then mixed thoroughly. After an incubation period of 10 minutes, the absorbance of the samples ($A_2$) and the reagent blank ($B_2$) was read at 340 nm. The change in absorbance for the sample ($\Delta A$) was calculated by subtracting its final absorbance ($A_2$) from its initial absorbance ($A_1$). The change in absorbance for the reagent blank ($\Delta B$) was determined by subtracting its final absorbance ($B_2$) from its initial absorbance ($B_1$). The triacylglycerol concentration in the sample was calculated as follows:

$$\text{Triacylglycerol (mg/l)} = \frac{(\Delta A - \Delta B) \times 885 \times 0.31}{6.22 \times 1.4 \times 0.05} - \text{free glycerol}$$

$\Delta A = (A_2 - A_1)$

$\Delta B = (B_2 - B_1)$

885 = Molecular weight of triacylglycerol expressed as triolein

0.31 = Total volume (ml)

6.22 = Molar absorptivity of NADH at 340 nm

1.4 = Correction factor allowing for light path and conversion to absolute optical density.

0.05 = Sample volume (ml)

## 2.6 PLASMA $\beta$-HYDROXYBUTYRATE ASSAY

Concentrations of plasma $\beta$-hydroxybutyrate were determined using an enzymatic method (Williams and Mellanby, 1974), which involved the enzymatic oxidation of hydroxybutyrate to acetoacetate.

$$\beta\text{-hydroxybutyrate} + NAD^+ \xrightarrow[\beta\text{-hydroxybutyrate dehydrogenase}]{} \text{Acetoacetate} + NADH + H^+$$

NADH absorbs at 340 nm, thus the increase in absorbance was directly proportional to the β-hydroxybutyrate concentration in the sample.

The assay incubation medium contained:

Hydrazine hydrate (1M)-glycine (0.3M) buffer, pH 8.5

NAD (10 mg/ml)

β-hydroxybutyrate dehydrogenase 15 units/ml

β-hydroxybutyrate standard, 1mM

**Procedure**

Plasma samples were deproteinised by adding an equal volume of perchloric acid (10 % w/v), then centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted and neutralised (pH 7-8) with potassium hydroxide (20 % w/v). The analysis was performed using a Titerteck analyser and 96-well plates. Distilled water (250 μl) was pipetted into the well nominated as the blank. To the well nominated as the standard was added 20 μl of β-hydroxybutyrate standard solution. Distilled water (20 μl) was added to the reagent blank. To the remaining wells was added 20 μl of deproteinised sample. Incubation medium (250 μl) was added to the standard, the reagent blank and samples, then mixed thoroughly. The absorbance of the samples ($A_1$), standard ($C_1$) and reagent blank ($B_1$) was read at 340 nm, using the well containing distilled water as a blank. β-hydroxybutyrate dehydrogenase (5 μl) was added to the standard, samples and reagent blank, then mixed thoroughly. After an incubation period of 90 minutes at 37°C, the

absorbance of the samples ($A_2$), standard ($C_2$) and the reagent blank ($B_2$) were determined at 340 nm. The β-hydroxybutyrate concentration in the sample was calculated as follows:

$$\beta\text{-hydroxybutyrate (mM)} = \frac{\Delta A - \Delta B}{\Delta C - \Delta B} \times \text{dil.}$$

$\Delta A = A_2 - A_1$

$\Delta B = B_2 - B_1$

$\Delta C = C_2 - C_1$

dil. = dilution factor for deproteinised sample

## 2.7 PLASMA ACETATE ASSAY

Concentrations of plasma acetate were determined using an enzymatic method based on that described by Bergmeyer and Mollering (1974).

**Principle**

1) Acetate + ATP + CoA $\xrightarrow[\text{synthetase}]{\text{Acetyl CoA}}$ Acetyl CoA + AMP

2) ATP + AMP $\xrightarrow{\text{Myokinase}}$ 2 ADP

3) Phosphoenolpyruvate + ADP $\xrightarrow[\text{kinase}]{\text{Pyruvate}}$ Pyruvate + ATP

4) Pyruvate + NADH $\xrightarrow[\text{dehydrogenase}]{\text{Lactate}}$ Lactate + $NAD^+$

NADH absorbs at 340 nm, thus the decrease in absorbance was directly proportional to the acetate concentration in the sample.

The assay incubation medium contained:

CoA, 0.4 mM

Tris, 50mM

$MgCl_2$, 10 mM

ATP, 3 mM

NADH, 0.25 mM

Glutathione, 0.1 mM

Myokinase, 4 units/ml

Pyruvate kinase, 8 units/ml

Lactate dehydrogenase, 12 units/ml

Acetate standard, 1 mM

**Procedure**

Plasma samples were deproteinised by adding an equal volume of perchloric acid (10 % w/v), then centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted and neutralised (pH 7-8) with potassium hydroxide (20 % w/v). The analysis was performed using a Titerteck analyser and 96-well plates. To the well nominated as the standard was added 10 $\mu$l acetate standard (1mM). Distilled water (250 $\mu$l) was pipetted into the well nominated as the blank. To the well nominated as the reagent blank was added assay incubation medium (250 $\mu$l). Distilled water (10 $\mu$l) was added to the reagent blank. To the remaining wells was added 10 $\mu$l deproteinised sample. Incubation medium (250 $\mu$l) was added to the standard and samples, then mixed thoroughly. The absorbance of the samples ($A_1$), standard ($C_1$) and reagent blank ($B_1$) was read at 340 nm, using the well containing distilled water as a blank. Acetyl CoA synthetase (5 $\mu$l) was added to the samples, standard and reagent blank. After an incubation period of 20 minutes at 37°C the absorbance of the sample ($A_2$), standard ($C_2$) and the reagent blank ($B_2$) were determined at 340 nm. The acetate concentration in the sample was calculated as follows:

$$\text{Acetate (mM)} = \frac{\Delta A - \Delta B}{\Delta C - \Delta B} \times \text{dil.}$$

$\Delta = A_2 - A_1$

$\Delta = B_2 - B_1$

$\Delta = C_2 - C_1$

dil. = dilution factor for deproteinised sample

## 2.8 MEASUREMENT OF GLUCOSE INCORPORATION INTO FATTY ACIDS IN RAT ADIPOSE TISSUE

On the day of the experiment rats were killed by cervical dislocation in a room adjacent to the room where they were housed. Epididymal adipose tissue was excised immediately, transferred into prewarmed Krebs-Ringer Bicarbonate buffer at 37 °C, and taken to the laboratory.

### Incubation

Adipose explants were prepared using spring-loaded ophthalmic iris scissors with a dissecting microscope as described by Dils and Forsyth (1981). Ultra-fine forceps were used to secure the tissue, whilst fine strips of adipose were dissected away and cut into 1 mg explants. Both the preparation and incubation of the explants was in gassed (95 % $O_2$: 5 % $CO_2$) Krebs Ringer-Bicarbonate buffer, pH 7.3 at 37°C containing 25 mM Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), 5.5 mM glucose and 2% BSA (Sigma, Poole, U.K.).

Adipose explants (8 per well) were transferred to multiwell culture plates (Flow Laboratories, Paisley, Scotland, U.K.) containing 1 ml of Krebs-Ringer Bicarbonate buffer, pH 7.3, with 5.5 mM glucose, and the appropriate concentration of the hormone under investigation (GIP or insulin). Explants were incubated with 0.125 $\mu$Ci D-(U-$^{14}$C)

glucose (Amersham International, Amersham, U.K.) for 2.5 hours, then washed with 1 ml Krebs-Bicarbonate buffer (pH 7.3) to remove the bulk of the radiolabelled glucose before subsequent lipid extraction.

**Lipid extraction and saponification**

The Folch Procedure was used for lipid extraction (Folch, Lees and Stanley, 1957). Adipose slices were blotted dry, weighed, and placed in quick-fit centrifuge tubes containing 2 drops 1M HCl, 8ml 1:1 chloroform/ methanol and 4 ml chloroform. Tubes were initially shaken to assist disintegration of the adipose tissue. After overnight extraction, 3ml of 0.88 % KCl was added, tubes shaken, and stoppers loosened to prevent excessive build up of pressure. The two phases were allowed to separate overnight, then the upper aqueous phase removed by aspiration and discarded. The lower chloroform phase containing total lipid was transferred into a glass scintillation vial and dried under air at 65°C on a heating block.

The samples were saponified by adding 4ml of ethanol: 60% KOH (3:1 v/v) containing 0.001% (w/v) Thymol blue (Sigma, Poole, U.K.). The vials were capped, shaken, and then placed on a heating block at 85°C for 2.5 hours. After saponification, the vials were cooled to room temperature and the samples acidified by the addition of 4ml 5M HCl (acidity was confirmed by the thymol blue turning from blue to pink). Unesterified fatty acids were removed by extracting twice with 4ml of petroleum ether (B.P. 40-60°C). The combined petroleum ether extracts were placed in a glass scintillation vial and dried under air at 65°C on a heating block. To each vial was added 10 ml of Optifluor Scintillant (Packard, Pangbourne, Berks, U.K.). This was whirlimixed, then placed on a scintillation counter (either a Packard Tri-Carb 460 or a LKB rackbeta 1218) and counted for 4 minutes.

Rates of fatty acid synthesis were determined from the extent of incorporation of radiolabelled glucose into saponifiable fatty acids (after total lipid extraction) and were expressed as nmol glucose/mg wet

weight of adipose tissue/hour.

## 2.9 MEASUREMENT OF ACETATE INCORPORATION INTO FATTY ACIDS IN OVINE ADIPOSE TISSUE

On the day of the experiment, the sheep were anaesthetized by intrajugular injection of 20 ml Sagatal (May and Baker, Dagenham, Essex, U.K.), after which they were exsanguinated. Samples of subcutaneous adipose tissue were excised from the flank and placed immediately in sterile 0.15M NaCl at 37°C as described by Vernon, Finley and Taylor (1985).

### Incubation

Explants weighing approximately 5 mg were prepared using scissors and incubated for one hour in Medium 199 (Gibco, Biocult Ltd, Paisley, Scotland), a buffered tissue culture medium containing Earles' Salts, L-glutamine, 25 mM Hepes, pH 7.3, 0.6 mM acetate and 5.5 mM glucose. The medium was supplemented with acetate (final concentration 2.6 mM), 0.6 mg/ml penicillin, 0.1 mg/ml streptomycin sulphate, 0.1 mg neomycin and 10 mg/ml BSA (0.15 mM) (all BSA used was essentially fatty acid free (Sigma, Poole, U.K.), and was dialysed before use).

Explants were incubated (8 per well) in multiwell culture plates (Flow Laboratories, Paisley, Scotland) with sodium (1-$^{14}$C)-acetate (Amersham International, Amersham, U.K.) for 24 hours in Medium 199 supplemented with the appropriate concentration of the hormone under investigation (GIP or insulin).

Rates of fatty acid synthesis were determined from the extent of incorporation of radiolabelled acetate into saponifiable fatty acids (after total lipid extraction), as described on page 48, and were expressed as nmol acetate/mg wet weight adipose tissue/hour.

# CHAPTER 3

## Studies of GIP secretion in pre-ruminant and young ruminant animals

## EXPERIMENT 3.1. EFFECTS OF INTRADUODENAL ADMINISTRATION OF NUTRIENTS ON GIP CONCENTRATION IN PORTAL BLOOD OF GOAT KIDS

### INTRODUCTION

Whilst it is recognised that in simple-stomached animals glucose and fat are the major stimuli for GIP secretion (Cataland *et al.*, 1974; Brown, Dryburgh, Moccia and Pederson, 1975 a), little is known concerning the regulation of GIP secretion in ruminant species where, in the adult animal, relatively small amounts of glucose and fat are absorbed (Leng, 1970; Byers and Schelling, 1988). These nutrients do, however, make a significant contribution to nutrient supply in the young milk-fed animal. Rumen development depends on the consumption of solid feeds and the establishment of a rumen microbial population (Phillipson, 1977). Prior to this ingested nutrients by-pass the rumen, flowing directly to the abomasum via the oesophageal groove, and are digested and absorbed from the small intestine. Therefore, whilst it might be anticipated that relationships between nutrient absorption and GIP secretion may be modified in the ruminant, responses in the pre-ruminant may resemble more closely those in simple-stomached animals.

An experiment was conducted in both pre-ruminant and young ruminant animals with the aim of determining GIP responses to nutrient absorption. Because of their potency in eliciting GIP secretion in simple-stomached animals, glucose and fat were selected for study in this initial experiment. In an attempt to ensure that GIP release would be detected even if the test nutrients were weak stimuli for GIP secretion, animals were surgically prepared, under anaesthesia, to facilitate intraduodenal administration of nutrients and withdrawal of blood from the portal vein, which receives blood directly from vessels draining the intestine.

## EXPERIMENTAL

### Animals and their management

A group of 12 male goat kids (British-Saanen) were taken from the nannies when 24 hours old and reared on goats' milk only, in order to delay normal development of rumen function (pre-ruminants). A second group of 14 goats remained with the nannies and had access to long hay, straw and concentrates (ruminants). The average ages of the pre-ruminant and ruminant animals when the experiments were conducted were 62 (Standard Error of the Mean (S.E.M.) = 0.8) and 64 (S.E.M. = 1.0) days respectively.

### Surgical preparation

After an overnight fast the animal was weighed and anaesthetised with pentobarbital sodium injected into a jugular vein. The animal was placed on its left side and a polyethene catheter (Internal diameter 0.88 mm; Dural Plastics, Dural, Australia) was inserted into a jugular vein for maintenance of anaesthesia by administration of pentobarbital sodium. An endotracheal tube was introduced to prevent inhalation of saliva.

The method of portal vein cannulation was similar to that described by Katz and Bergman (1969). An incision of approximately 12 cm was made behind and parallel to the last rib. Muscle layers and peritoneum were dissected to expose the pyloric sphincter, and to allow a length (approximately 15 cm) of small intestine to be exteriorised temporarily. A vinyl catheter (Internal diameter 0.58 mm; Dural Plastics, Dural, Australia) was inserted into a mesenteric vein and threaded along the portal vein until the tip could be felt at the point where the vein entered the liver. In this and all subsequent experiments catheters for blood sampling were flushed and filled with a sterile solution of 0.9 % (w/v) NaCl and 0.5 % (w/v) $Na_3C_6H_5O_7.2H_2O$ to maintain patency of the catheter between sampling. The portal catheter

was secured with sutures and the intestine returned to the body cavity, ensuring the pyloric sphincter was accessible. The area was covered throughout the experiment with warm damp swabs.

**Experimental procedure**

Three samples of portal blood (2.5 ml) were withdrawn prior to nutrient injection. A nutrient load pre-warmed to 37°C consisting of 30 ml of either double cream (49 % fat) or glucose solution (50 % w/v), was injected via a needle (18 Gauge) inserted into the lumen of the duodenum approximately 2 cm after the pyloric sphincter. Further blood samples were taken at 10-minute intervals up to 90 minutes after injection. Samples for measurement of glucose and triacylglycerol concentrations were mixed immediately with heparin (10 units/ml), centrifuged at 8800 *g* for 5 minutes in a benchtop centrifuge (Eppendorf, Hamburg, Germany), plasma removed and stored at -20°C until analysed as described in Chapter 2. Blood taken for the measurement of GIP concentrations was mixed with 1000 KIU aprotinin/ml (Sigma, Poole, U.K.), allowed to clot at room temperature for 2.5 hours, centrifuged, serum removed and stored at -20°C until analysed as described in Chapter 2.

**Statistical analysis**

Data are given as means with Standard Error of the Difference (S.E.D.). Statistical analysis was by analysis of variance and significance was determined by variance ratios and t-tests.

## RESULTS

As intended, even though pre-ruminant goats were maintained on a milk-only diet beyond the time at which solid feeds would normally have been introduced, growth rates were comparable to those of the ruminants during the 9 weeks prior to the experiment. Use of the

animals at this age allowed maximum development of the rumen in the ruminant goats before weight differences between the two animal groups could become a confounding factor in the experiment. The mean live weights for the pre-ruminant and ruminant groups were 12.3 kg (S.E.M. = 0.59) and 13.6 kg (S.E.M. = 0.43) respectively.

**Responses of pre-ruminant goat kids**

In pre-ruminant goats, intraduodenally-administered fat was absorbed giving rise to an increase in plasma triacylglycerol throughout the 90-minute sampling period (Fig. 3.1.a). Intraduodenal injection of a glucose load increased the plasma glucose concentration from a basal level of approximately 4 mM to a maximum of 8 mM within 70 minutes of nutrient injection (Fig. 3.1.b). Glucose concentrations remained at the maximum level for the remainder of the sampling period.

The increase in plasma triacylglycerol concentration was associated with a significant rise ($P < 0.05$) in portal GIP concentrations (Fig. 3.1.c). Although intraduodenally-administered glucose was absorbed, giving an increase in plasma glucose concentration, there was no GIP response (Fig. 3.1.c). In fact, portal GIP concentrations were moderately decreased from basal levels by 90 minutes after the intraduodenal glucose load ($P < 0.05$).

**Responses of ruminant goat kids**

In ruminant goats, intraduodenal injection of a fat load led to a gradual increase in plasma triacylglycerol throughout the 90-minute sampling period (Fig. 3.2.a). Intraduodenal administration of glucose resulted in an increase in plasma glucose concentration comparable to that observed in pre-ruminant goats (Fig. 3.2.b).

The increase in plasma triacylglycerol concentration was associated with a significant rise ($P < 0.05$) in portal GIP concentrations (Fig. 3.2.c). Intraduodenally-administered glucose increased plasma glucose concentration, but, as was the case for the pre-ruminants, there

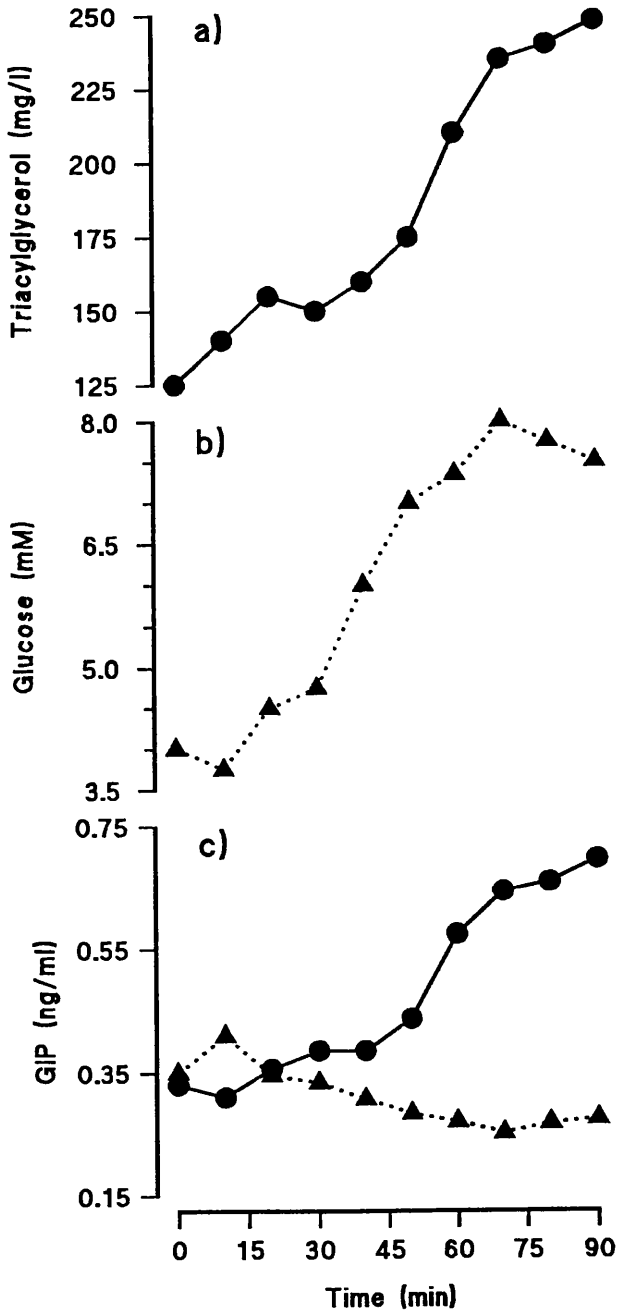



**Figure 3.1.** Portal concentrations of (a) plasma triacylglycerol, (b) plasma glucose and (c) serum GIP in pre-ruminant goat kids given intraduodenally-administered fat (●—●) or glucose (▲---▲). Data are means of 6 animals. S.E.D. for triacylglycerol, glucose, and GIP were 24.8 mg/l, 0.642 mM and 0.043 ng/ml respectively.

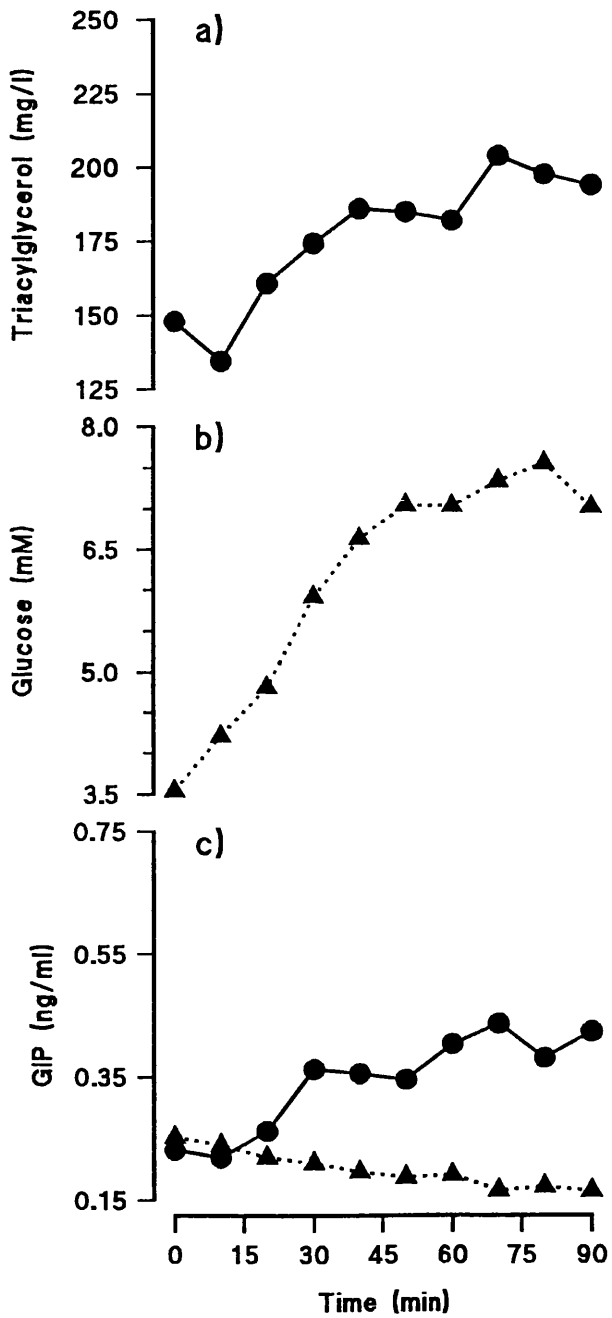



**Figure 3.2.** Portal concentrations of (a) plasma triacylglycerol, (b) plasma glucose and (c) serum GIP in ruminant goat kids given intraduodenally-administered fat (●—●) or glucose (▲----▲). Data are means of 7 animals. S.E.D. for triacylglycerol, glucose, and GIP were 21.06 mg/l, 0.488 mM and 0.044 ng/ml respectively.

was no GIP response (Fig. 3.2.c). By the end of the sampling period portal GIP concentrations had declined slightly ($P < 0.05$).

## DISCUSSION

Intraduodenal injection of nutrients prevented nutrient modification in the rumen and avoided complications associated with effects on gastric emptying, which might otherwise have influenced GIP secretion (Creutzfeldt *et al.*, 1978). Direct portal sampling avoided dilution of secreted GIP within the general circulation and the possibility of hepatic clearance of GIP, though GIP extraction by the liver is considered negligible (Hanks, Anderson, Wise, Putnam, Meyers and Jones, 1984). In simple-stomached animals, GIP secretion is dependent upon the absorption of nutrients. In both pre-ruminant and ruminant goats, intraduodenal administration of fat or glucose gave rise to marked changes in metabolite concentrations in portal blood, which confirmed that nutrient absorption had occurred. The absorption of fat, and its passage into the general circulation via the lymphatic system, was associated with significant increases in GIP concentrations in portal blood. In contrast, glucose absorption did not appear to elicit GIP secretion in either pre-ruminant or ruminant animals. These results demonstrate that in pre-ruminant and young ruminant animals, as in simple-stomached animals (Cataland *et al.*, 1974) fat is a potent GIP secretagogue.

It appears that differences exist between ruminants and non-ruminants with regard to the ability of glucose to elicit GIP secretion. It is especially surprising that intraduodenal administration of glucose, a major GIP secretagogue in simple-stomached animals (Anderson *et al.*, 1978), did not induce GIP secretion in the pre-ruminant animals, where lactose makes a considerable contribution to the dietary energy supply and where glucose, derived by hydrolysis of lactose, is efficiently absorbed; maintaining animals on a milk diet

is known to prevent the normal decline in intestinal $Na^+$/glucose cotransport (Shirazi-Beechey, Hirayama, Wang, Scott, Smith and Wright, 1991).

However, the possibility that anaesthesia or surgical intervention used in this experiment may have influenced GIP secretion directly, or indirectly by affecting the absorption of nutrients, can not be excluded. Furthermore, serum degradation of GIP could possibly account for an underestimation of circulating GIP concentrations.

## EXPERIMENT 3.2. EFFECTS OF CONSUMPTION OF MILK AND MILK CONSTITUENTS ON CIRCULATING CONCENTRATIONS OF GIP IN PRE-RUMINANT ANIMALS

## INTRODUCTION

The results of Experiment 3.1 indicate that in the pre-ruminant animal fat absorption elicits GIP release but that, even prior to the development of rumen function, intestinal absorption of glucose fails to stimulate GIP secretion. As this contrast with simple-stomached animals (Cataland *et al.*, 1974) is especially surprising, given that the milk-fed animal consumes and digests large amounts of lactose, it was decided to investigate the GIP response to glucose, and to other nutrients, under more physiologically-normal conditions.

Three experiments were conducted in conscious, meal-fed pre-ruminant animals, with the objectives of (a) making a preliminary assessment of the effect of milk consumption on concentrations of GIP in the general circulation, (b) identifying specific nutrients that elicit GIP secretion, and (c) determining whether there is a GIP response to feeding in the newborn animal.

## EXPERIMENTAL

### EXPERIMENT 3.2.a. THE EFFECT OF MILK INGESTION ON CIRCULATING CONCENTRATIONS OF GIP IN THE CALF

#### Animals and their management

A study was conducted in five 33-day-old calves. The animals were penned individually and received 3 l of a proprietary cows' milk replacer from a bucket at 09.00 hours each day.

**Experimental procedure**

Blood was withdrawn from a jugular vein directly into evacuated heparin-treated glass tubes through a small bore (22 Gauge) needle (Vacutainer; Becton-Dickinson Ltd, Wembley, U.K.) immediately prior to milk ingestion and again 1 hour post-feeding. Blood samples were mixed with aprotinin (1000 KIU /ml), centrifuged at 1300 *g* for 15 minutes at 4°C, plasma removed and stored at -20°C until analysed for GIP as described in Chapter 2.

**Statistical analysis**

Differences between pre-feed and post-feed GIP concentrations were assessed for statistical significance using Students t-test for paired observations.

EXPERIMENT 3.2.b. EFFECT OF CONSUMPTION OF MILK AND MILK CONSTITUENTS ON CIRCULATING CONCENTRATIONS OF GIP IN PRE-RUMINANT GOAT KIDS

**Animals and their management**

Fifteen male British-Saanen goat kids were used. Kids were removed from their mothers within 48 hours of birth and were housed in pens in groups of 5 animals. Animals were bedded on sawdust and were group-fed from a bowl, receiving goats' milk only in amounts increasing to 2 l/goat per day at about 7 days of age. Milk was given in 2 equal meals at 09.00 and 15.00 hours. The experiment was started when the animals reached 48 days (S.E.M. = 2) of age and was completed within 8 days.

**Experimental procedure**

On the day prior to the experiment a polyethylene catheter (Internal diameter 0.5 mm; Dural Plastics, Dural, Australia) was inserted into a jugular vein for the withdrawal of blood samples. To

avoid disruption to normal feeding behaviour, the animals remained in their pens and were group-fed during the experiment. It was not therefore possible to monitor intakes of individual animals, but animals were of a similar age and size and all drank readily, apparently consuming approximately equal amounts. On 3 occasions animals did not consume the test meal offered, but sampling was continued to obtain control data.

Test meals consisted of 1 l of goats' milk (n=13), 1 l of skimmed goats' milk (n=12), 0.5 l of solutions of cows' milk fat (40 g/l (80 ml double cream + 420 ml water); n=7), of lactose (122 mM; n=4), of glucose (122 mM; n=3) or of casein (as sodium caseinate) plus lactose (30 g/l and 122 mM respectively; n=3). To achieve a composition similar to that of whole milk with respect to major minerals (Jenness, 1974) the following were added to each solution: $Na_3C_6H_5O_7$ (5 mM), $CaCl_2$ (5.5 mM), $NaH_2PO_4$(10 mM), KCl (18 mM), $K_2SO_4$ (9 mM) and $MgSO_4$ (0.5 mM). It was considered necessary to restrict the volume and amounts of individual milk constituents given to approximately 50 % of those consumed in milk in order to avoid digestive upset.

Blood samples (2.5ml) were withdrawn at 08.50 and 08.55 hours and a test meal was given at 09.00 hours. Further samples were taken 15, 30, 45 and 60 minutes after feeding and thereafter at hourly intervals for a total of 5 hours or, when lactose or glucose was given, for 3 hours. Samples were mixed immediately with heparin (10 units/ml) and aprotinin (1000 KIU/ml), centrifuged at 8800 *g* for 5 minutes in a benchtop centrifuge (Eppendorf, Hamburg, Germany) and the plasma stored at -20°C until analysed for GIP, glucose and triacylglycerol as described in Chapter 2.

**Statistical analysis**

Data are given as means with S.E.D. Values for the two pre-feed samples were averaged and the means presented as time = 0. GIP and metabolite concentrations were compared within and between test meals

by analysis of variance of paired data and statistical significance was determined by variance ratios and paired t-tests. Overall response to each test-meal was assessed using data from all animals. Integrated mean change in concentration from pre-feed values was calculated for individual animals from area under the response curve and expressed on an hourly basis. These values were then meaned and subjected to analysis of variance. The rate of increase in GIP secretion was estimated by the slope from the regression of GIP against time during the first 30 minutes after nutrient ingestion.

## EXPERIMENT 3.2.c. EFFECT OF SUCKLING ON GIP CONCENTRATION IN PORTAL BLOOD OF NEWBORN GOAT KIDS.

### Animals and experimental procedure

Six British-Saanen goat kids were used in the experiment. Immediately after birth and before suckling, 10 cm of polyethylene tubing (Internal diameter = 0.5 mm, External diameter = 0.9 mm; Dural Plastics, Dural, Australia) containing heparinised physiological saline (2 units/ml) was placed in the umbilical vein and threaded along until it reached the abdominal wall to allow sampling of portal venous blood. Blood samples were taken before and at intervals for four hours after the beginning of ingestion of colostrum, which was taken directly from the nanny or via a bottle. Blood was mixed immediately with heparin (10 units/ml) and aprotinin (1000 KIU/ml), centrifuged at 8800 *g* for 5 minutes in a benchtop centrifuge (Eppendorf, Hamburg, Germany), plasma removed and stored at -20°C until analysed for GIP as described in Chapter 2.

### Statistical analysis

Data are given as means with S.E.D. Statistical analysis was by one-way analysis of variance and significance was determined by

variance ratios and t-tests.

## RESULTS

### EXPERIMENT 3.2.a. THE EFFECT OF MILK INGESTION ON CIRCULATING CONCENTRATIONS OF GIP IN THE CALF

As shown in Figure 3.3., in calves given their normal feed, circulating GIP concentrations increased significantly ($P < 0.01$) from a mean basal level of 0.6 ng/ml (S.E.M. = 0.1) to 1.5 ng/ml (S.E.M. = 0.3) 1 hour after feeding.

### EXPERIMENT 3.2.b. EFFECT OF CONSUMPTION OF MILK AND MILK CONSTITUENTS ON CIRCULATING CONCENTRATIONS OF GIP IN PRE-RUMINANT GOAT KIDS

#### Responses to milk

Plasma concentrations of glucose, triacylglycerol, and GIP in pre-ruminant goat kids before and after milk consumption are shown in Figure 3.4 (a, b and c, respectively). Glucose concentration increased ($P < 0.01$) within 15 minutes of milk consumption and after a slight, transient fall remained higher than basal (at least $P < 0.01$) for 4 hours after feeding. There were no significant changes in plasma concentration of triacylglycerol during the 5 hour sampling period, though concentrations tended to increase during the later stages. Mean plasma acetate concentration before feeding was 0.25 mM and at subsequent sampling times was 0.22, 0.24, 0.23, 0.26, 0.25, 0.27, 0.26, and 0.24 (S.E.D. = 0.069) mM.

Plasma GIP concentration increased rapidly during the first 30 minutes at a rate of 13.8 pg/min (S.E.M. = 4.2) and was markedly higher than basal ($P < 0.001$) within 45 minutes of milk consumption, and remained so throughout the sampling period.

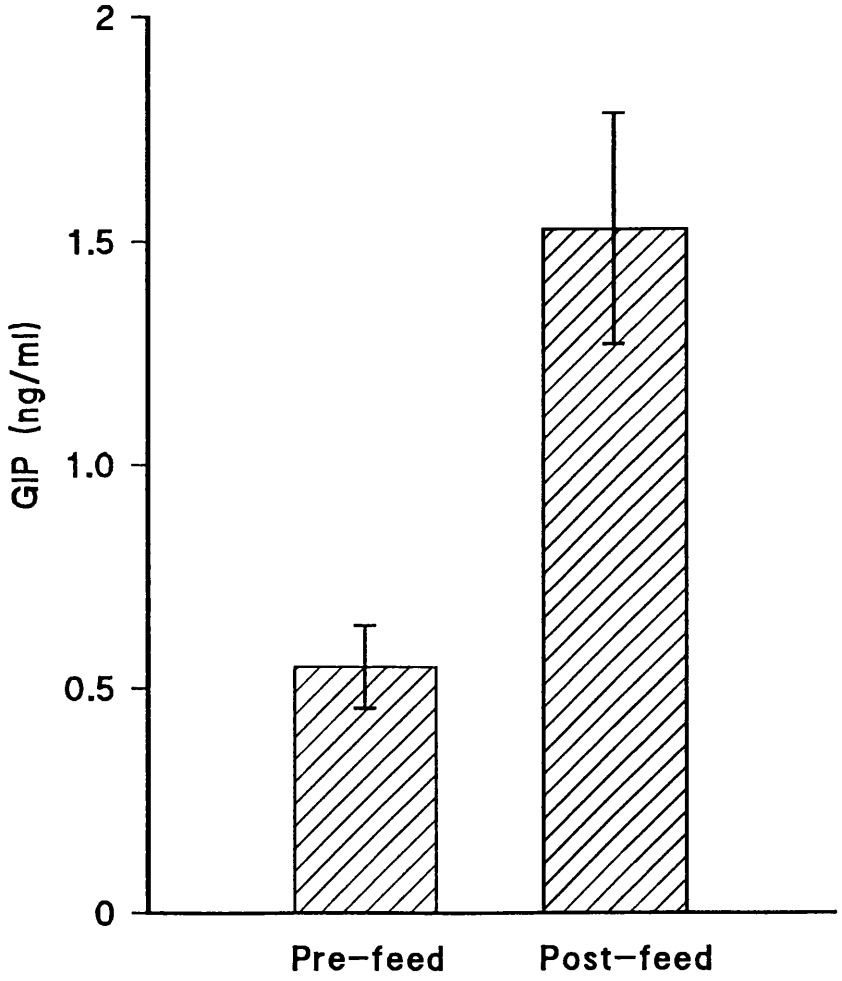



**Figure 3.3.** The response of plasma GIP concentration to ingestion of 3 l of milk in the calf. Data are means with S.E.M. of 5 animals.

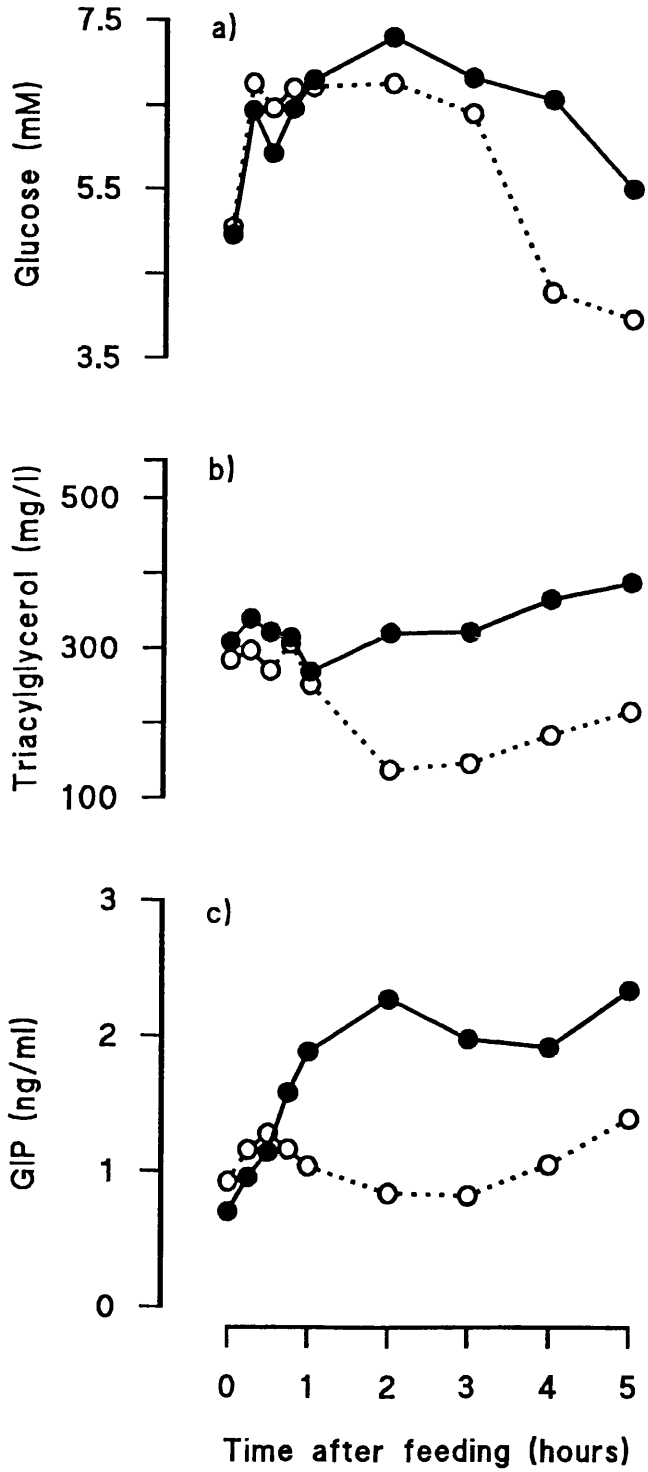



**Figure 3.4.** Plasma concentrations of (a) glucose, (b) triacylglycerol and (c) GIP in pre-ruminant goat kids given milk (●—●) or skimmed milk (○---○). Data are means of 11 animals. S.E.D. within feeds are 0.55 mM, 45.1 mg/l and 0.189 ng/ml respectively, and between feeds are 0.59 mM, 46.6 mg/l and 0.221 ng/ml.

**Response to skimmed milk**

Consumption of skimmed milk increased ($P < 0.05$) plasma glucose concentration within 15 minutes (Figure 3.4.a). The increase was maintained for 3 hours, after which glucose concentration declined rapidly, falling below the basal concentration ($P < 0.05$) by 5 hours after feeding. The relatively low concentration of fat in the skimmed milk was reflected in reductions ($P < 0.05$) in plasma triacylglycerol concentration within 2 hours of feeding (Figure 3.4.b). Although concentrations tended to increase thereafter, they did not approach basal levels until the end of the sampling period.

There was a slight, non-significant ($P > 0.05$) increase in plasma GIP concentration during the 30 minutes after feeding, with a rate of 11.8 pg/min (S.E.M. = 4.3), but GIP concentrations were otherwise unaffected except for a small increase ($P < 0.05$) 5 hours after feeding (Figure 3.4.c).

**Response to cream**

As shown in Figure 3.5, responses to milk in the five animals which on a separate occasion received milk fat, as cream, were typical of the general pattern observed after milk ingestion. When cream was given plasma glucose concentration did not alter from basal (Figure 3.5.a), whereas triacylglycerol concentrations were increased ($P < 0.05$) 2 and 3 hours after feeding (Figure 3.5.b).

Plasma GIP concentration increased ($P < 0.01$) within 30 minutes, at a rate of 34.2 pg/min (S.E.M. =5.4) and remained elevated during the 4 hours after feeding (Figure 3.5.c).

**Response to lactose, glucose, or casein with lactose**

Plasma glucose concentrations increased after ingestion of solutions containing sugars, whereas plasma triacylglycerol concentrations changed little after feeding. In general, GIP concentrations were not significantly affected when lactose, glucose or

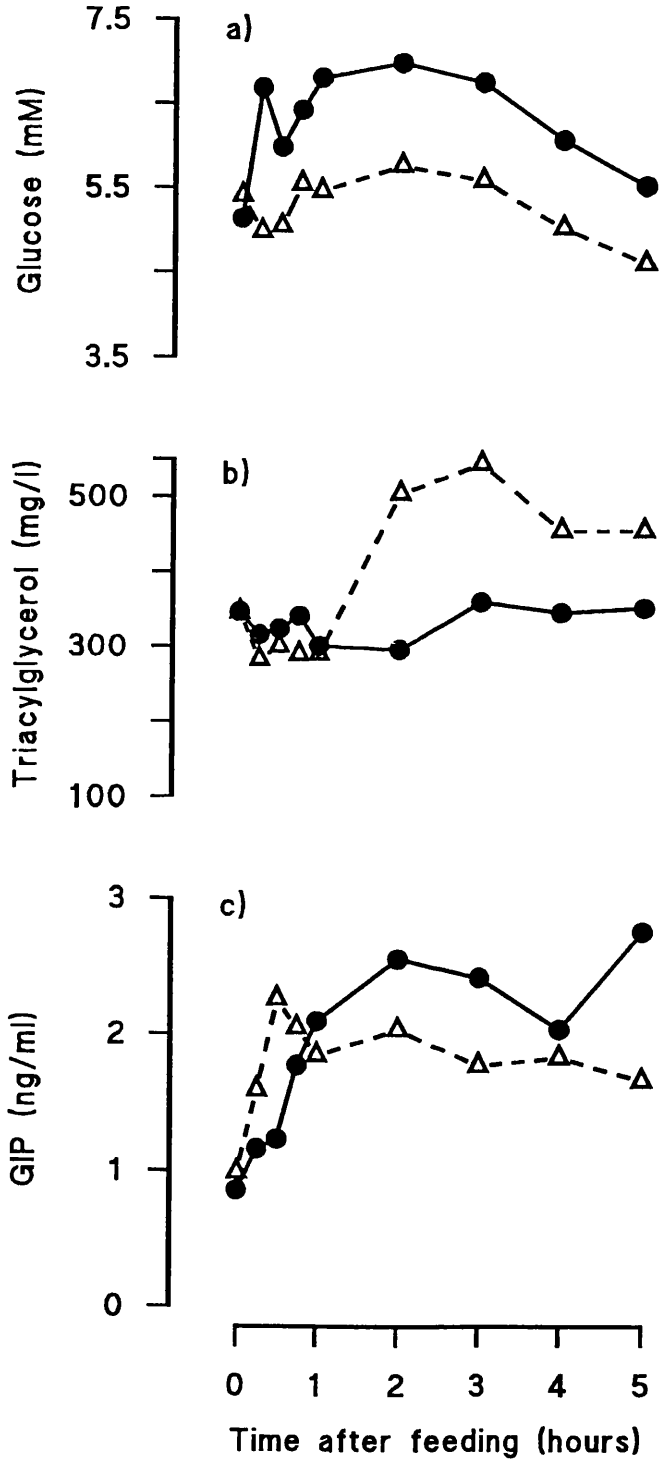



**Figure 3.5.** Plasma concentrations of (a) glucose, (b) triacylglycerol and (c) GIP in pre-ruminant goat kids given milk (●—●) or cream (▵---▵). Data are means of 5 animals. S.E.D. within feeds are 0.55 mM, 78.0 mg/l and 0.375 ng/ml respectively, and between feeds are 0.64 mM, 93.2 mg/l, and 0.442 ng/ml.

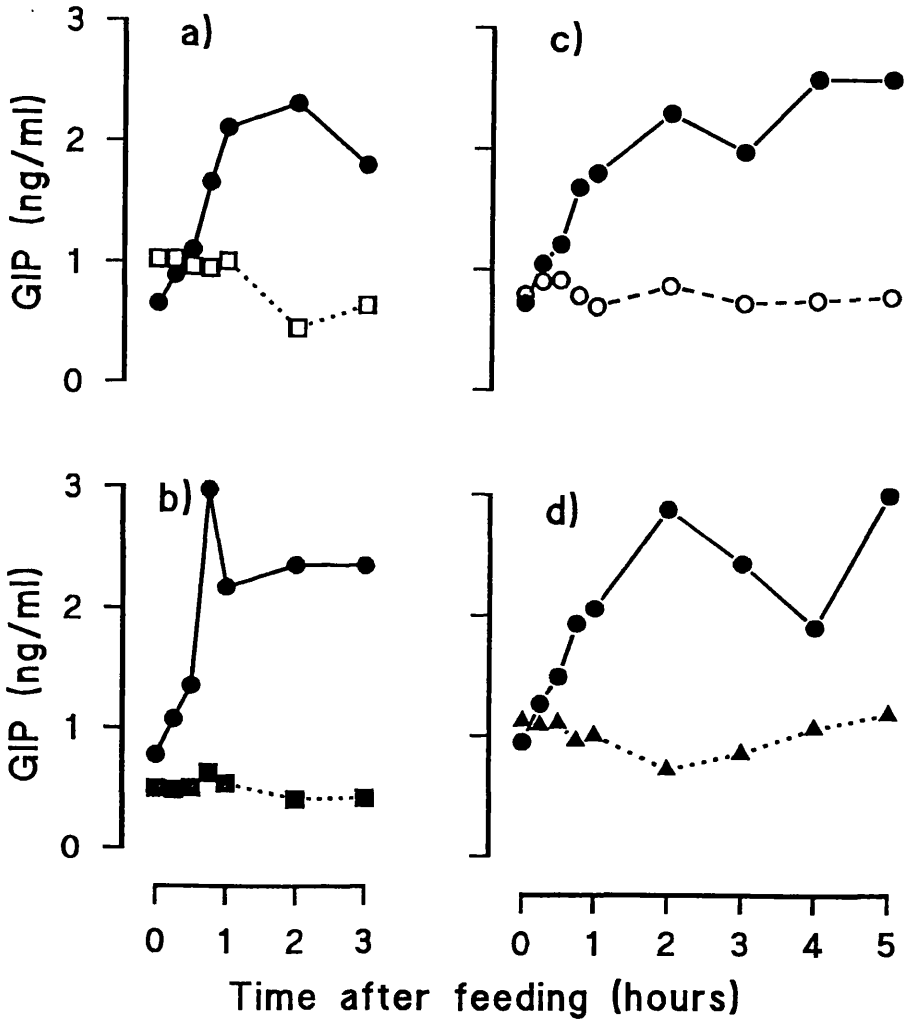



**Figure 3.6.** Plasma concentrations of GIP in goat kids given (a) milk (●—●) or, on a separate occasion, lactose (□···□); n=4, (b) milk (●—●) or glucose (■···■); n=3, (c) milk (●—●) or casein + lactose (○···○); n=3, and (d) milk (●—●) or no feed (▲···▲); n=3. Values are means with S.E.D., within and between feeds respectively, of (a) 0.204 and 0.217, (b) 0.333 and 0.433, (c) 0.292 and 0.314, and (d) 0.378 and 0.431 ng/ml.

**Table 3.1.** Integrated mean changes in the concentrations of GIP, glucose and triacylglycerol over a five hour period in pre-ruminant goat kids fed test meals.

| | milk | skimmed milk | cream | casein + lactose | no feed | SED (range) |
|---|---|---|---|---|---|---|
| GIP (ng/ml) | *** 1.17[a] | 0.05[b] | ** 0.85[a] | -0.030[b] | -0.17[b] | 0.32 - 0.15 |
| Glucose (mM) | *** 1.68[a] | ** 0.89[b] | -0.54[c] | 1.18[ab] | -0.76[c] | 0.74 - 0.16 |
| Triacylglycerol (mg/l) | -8.3[a] | ** -90.5[b] | ** 176.8[c] | -3.8[ab] | 28.4[a] | 72 - 35 |
| n | 13 | 12 | 7 | 3 | 3 | |

Mean changes which are significantly different from zero are * $P<0.05$, ** $P<0.01$, *** $P<0.001$

[abc]Means having different superscripts are significantly different ($P<0.05$).

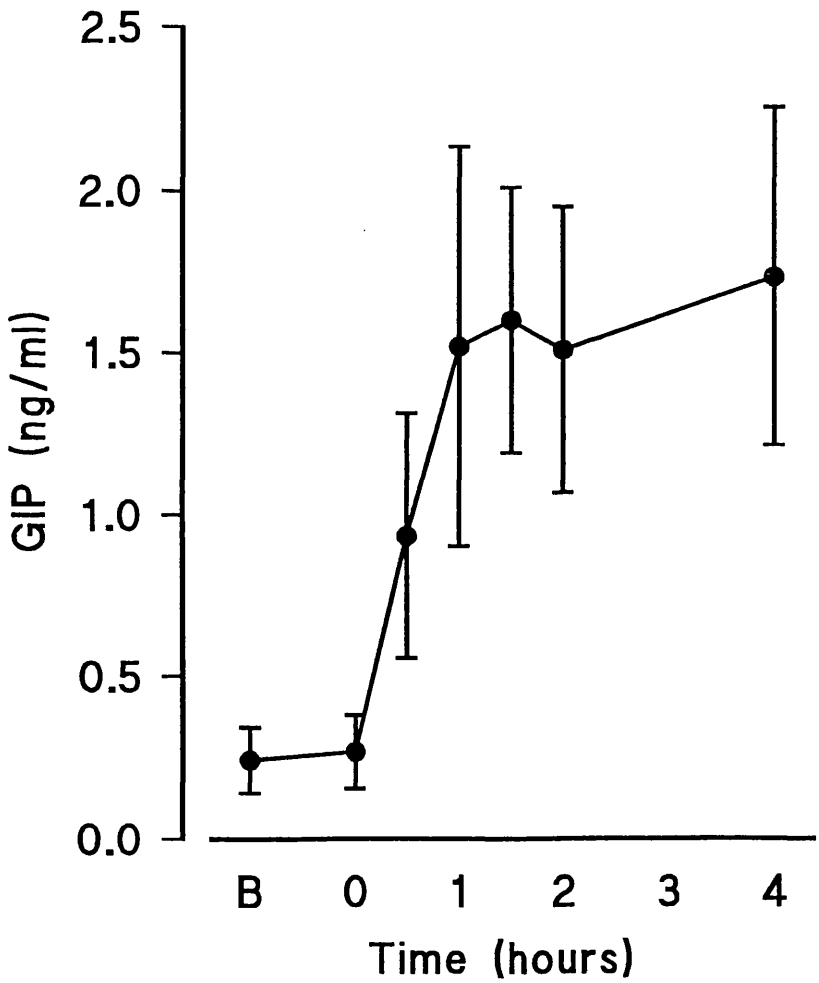



**Figure 3.7.** Concentration of GIP in portal venous plasma of goat kids in the first hour after birth (B), before suckling (0), and 4 hours after the beginning of suckling. Data are means, with S.E.M., of 6 animals.

casein with lactose were given (Figure 3.6). The reduced ($P<0.01$) concentration 2 hours after lactose consumption resembled the effect of giving no feed.

**Comparison of GIP responses to different feeds**

Post-prandial responses in GIP concentration were significantly greater for milk than for skimmed milk within 45 minutes of feeding ($P<0.01$). GIP concentration continued to rise in response to milk, but was unchanged after skimmed milk, and the difference in GIP response between these two feeds was highly significant ($P<0.001$) from 1 to 5 hours after feeding.

The time course of the GIP response to cream differed from that to milk. GIP concentration increased more rapidly after cream consumption, and was higher ($P<0.05$) than for milk 30 minutes after feeding. Subsequently GIP concentrations were similar for the two meals until 5 hours after feeding when plasma GIP concentration was lower ($P<0.05$) for cream than for milk. Overall GIP responses, in terms of integrated mean change in GIP concentration (Table 3.1), were significant only after the consumption of milk ($P<0.001$) and cream ($P<0.01$) and did not differ between these feeds.

## EXPERIMENT 3.2.c. EFFECT OF SUCKLING ON GIP CONCENTRATION IN PORTAL BLOOD OF NEWBORN GOAT KIDS

Figure 3.7. shows the change in portal GIP concentration in newborn goats during the 4 hour period after the initiation of suckling. The mean basal GIP concentration was 0.28 ng/ml. Each animal suckled within 1 hour of birth and by 90 minutes after feeding the plasma GIP concentration had reached 1.60 ng/ml ($P<0.02$).

## DISCUSSION

Previous studies in surgically-prepared goat kids (Experiment 3.1) showed that GIP is secreted into the portal vein in response to intraduodenally-administered nutrients. Experiment 3.2.a. demonstrates that GIP release in response to feeding leads to readily-detectable increases in GIP concentration in the general circulation. The marked GIP response within 1 hour of feeding in the pre-ruminant calves was consistent with rapid flow of milk through the oesophageal groove to the abomasum, and subsequent passage of nutrients to the duodenum. Basal GIP concentrations and the response to feeding were comparable to those seen in simple-stomached species (Pederson, Dryburgh and Brown, 1975; Schulz *et al.*, 1981).

Similarly, the results of Experiment 3.2.b show a marked post-prandial increase in circulating concentration of GIP in the milk-fed pre-ruminant goat kid. The virtual abolition of a GIP response to feeding when skimmed rather than whole milk was given, together with the marked response to cream, strongly suggest that fat was the major, if not the only, milk constituent to stimulate GIP secretion. The relatively small increase in GIP concentration 5 hours after skimmed milk ingestion possibly reflects absorption of the small amount of fat consumed, or of fat released from remnants of previous meals; it is likely that in the virtual absence of fat a relatively firm protein clot would be formed in the abomasum, slowing the release and passage of fat to the duodenum (Petit, Ivan and Brisson, 1987). Conversely, when cream was given no clot would have formed, allowing rapid passage of fat to the small intestine, which presumably explains the relatively rapid GIP response to fat alone as compared with that to milk.

Because of effects of clot formation, and of factors such as meal size and fat concentration on rate of abomasal emptying (Ash, 1964), and because different amounts of milk constituents were given, patterns of absorption of individual nutrients probably differed slightly

depending on whether they were given singly or in combination. Therefore, it is not possible to explain the entire GIP response to milk in terms of fat absorption. However, the results clearly demonstrate that carbohydrate, whether lactose or glucose, does not elicit GIP secretion. The possibility that the goat kids had developed a degree of rumen function and that dietary carbohydrate had in fact been fermented prior to the small intestine can be discounted because increases in plasma glucose concentrations within 15 minutes of consumption of milk, skimmed milk or solutions containing lactose or glucose were entirely consistent with rapid passage of nutrients to the abomasum and carbohydrate absorption from the small intestine. Furthermore, plasma acetate concentrations were unchanged after milk consumption, indicating the absence of microbial activity and VFA production in the rumen. Thus, the pre-ruminant differs from simple-stomached animals (Cataland *et al.*, 1974) in failing to secrete GIP in response to carbohydrate absorption.

Products of protein digestion may have contributed to the stimulation of GIP secretion, although amino acid absorption is a relatively weak stimulus for GIP secretion in simple-stomached animals (O'Dorisio *et al.*, 1976). The slight, transient increase in GIP concentrations after skimmed milk consumption may have been evoked by the absorption of whey proteins which, along with lactose, flow rapidly and independently of clot formation to the duodenum (Petit *et al.*, 1987). Also, absorption of protein, peptides or amino acids as well as fat may have contributed to the rise in GIP concentration 5 hours after the skimmed milk meal. However, an effect of protein on GIP release was not evident when casein and lactose were given.

These results confirm in conscious animals the previous observations in anaesthetised pre-ruminant goats (Experiment 3.1) that glucose absorption does not elicit GIP secretion, and that fat is a potent stimulus for GIP release. The large increase in plasma GIP concentration after drinking milk indicates a physiological role for GIP

in pre-ruminants.

In the newborn goat (Experiment 3.2.c), concentrations of GIP in portal blood immediately after birth and before suckling were within the same range as basal levels in older pre-ruminant goats (Experiment 3.2.b). Also, the GIP response brought about by suckling colostrum for the first time was of the same magnitude as that following milk consumption in older goat kids. Thus GIP, in common with the other gastrointestinal hormones gastrin and CCK (Guilloteau *et al.*, 1992), is responsive to ingestion of the first meal in pre-ruminant animals.

## EXPERIMENT 3.3. EFFECT OF CONSUMPTION OF MILK, MILK CONSTITUENTS AND CONCENTRATES ON CIRCULATING CONCENTRATIONS OF GIP IN RUMINANT GOAT KIDS

## INTRODUCTION

The results of Experiment 3.2 confirmed that fat is a potent GIP secretagogue in the pre-ruminant animal, and that glucose absorption is ineffective in eliciting GIP secretion. The aim of the present study was to determine whether GIP responses to nutrient absorption are modified after the development of rumen function. To do this, the ability of different milk constituents to induce GIP secretion was evaluated in young ruminant goat kids. As a preliminary to studying GIP secretion in the adult ruminant, studies were conducted also to determine whether circulating GIP levels in the young goat kids were responsive to ingestion of concentrates and to evaluate the potential GIP-releasing effect of the type of protein contained within the concentrates.

## EXPERIMENTAL

### Animals and their management

Twelve male British Saanen goat kids were used. Kids were removed from their mothers within 48 hours of birth and were housed in pens in groups of 3 to 5 animals. Animals were bedded on sawdust and were group-fed from a bowl, receiving goats' milk in amounts increasing to 2 l/goat per day at about 7 days of age, with access to long hay, straw and concentrates. Milk was given in 2 equal meals at 09.00 and 15.00 hours for the first 6 weeks, then reduced to 1 l per day, given at 09.00 hours, thereafter. The experiment was conducted when the kids reached an age of 64 days (S.E.M. = 2).

**Experimental procedure**

The experimental procedure was essentially the same as in Experiment 3.2.b. On the day prior to the experiment a polyethene catheter (Internal Diameter 0.5 mm; Dural Plastics, Dural, Australia) was inserted in a jugular vein for the withdrawal of blood samples. The animals were group-fed in their pens during the experiment and appeared to consume approximately equal amounts.

Test meals consisted of 1 l of goats' milk (n=12), 1 l of skimmed goats' milk (n=12), 0.5 l of solutions of cows' milk fat (40 g/l (80 ml double cream + 420 ml water); n=5), of lactose (122 mM ; n=4), of glucose (122 mM; n=3), of casein (as sodium caseinate) (30 g/l; n=4), of casein (as sodium caseinate) and lactose (30 g/l and 122 mM respectively; n=8), of casein hydrolysate and lactose (30 g/l and 122 mM respectively; n=4) and of soya protein isolate and lactose (30 g/l and 122 mM respectively; n=4). Minerals were added to achieve a composition similar to that of whole milk, as described in Experiment 3.2.b. The volume and amounts of individual milk constituents given were restricted to approximately 50 % of those consumed in milk in order to avoid digestive upset. A further test meal consisted of approximately 150 g concentrates, followed by *ad libitum* access to hay. On 9 occasions control samples were obtained from animals given no feed.

Blood samples (2.5 ml) were taken at 08.50 and 08.55 hours and a test meal was given at 09.00 hours. Further samples were taken at 15, 30, 45 and 60 minutes after feeding and thereafter at hourly intervals for a total of 5 hours or, when lactose or glucose was given for 3 hours. Blood samples were taken hourly for 5 hours after the concentrates meal. Samples were mixed immediately with heparin and aprotinin (1000 KIU/ml), centrifuged at 8800 *g* for 5 minutes in a benchtop centrifuge (Eppendorf, Hamburg, Germany) and plasma stored at -20°C until analysed for GIP, glucose and triacylglycerol as described in Chapter 2.

### Calculations and statistics

Data are given as means with S.E.D. Values for the two pre-feed samples were averaged and the means presented at time = 0. GIP and metabolite concentrations were compared within and between test meals by analysis of variance of paired data and statistical significance was determined by variance ratios and paired t-tests. Overall response to each test meal was assessed using data from all animals. Integrated mean change in concentration from pre-feed values was calculated for individual animals from area under the response curve and expressed on an hourly basis. These values were then meaned and subjected to analysis of variance.

## RESULTS

### Responses to milk

Plasma concentrations of glucose, triacylglycerol and GIP in 12 ruminant goat kids before and after milk consumption are shown in Figure 3.8 (a, b and c respectively). Plasma glucose concentration increased ($P < 0.001$) within 15 minutes of milk ingestion and remained higher than basal ($P < 0.001$) for 5 hours after feeding. Plasma triacylglycerol concentration increased significantly ($P < 0.01$) within 2 hours and continued to rise for the duration of sampling ($P < 0.001$). Plasma GIP levels increased ($P < 0.05$) within 15 minutes and were markedly elevated ($P < 0.001$) from 45 minutes, and remained so throughout the sampling. Mean plasma acetate concentration before feeding was 0.46 mM and at subsequent times was 0.43, 0.45, 0.45, 0.44, 0.42, 0.47, 0.42 and 0.39 (S.E.D. = 0.041) mM.

### Response to skimmed milk

Consumption of skimmed milk increased ($P < 0.01$) plasma glucose concentration above basal within 15 minutes (Figure 3.8.a). The increase was maintained for the duration of sampling. There was a

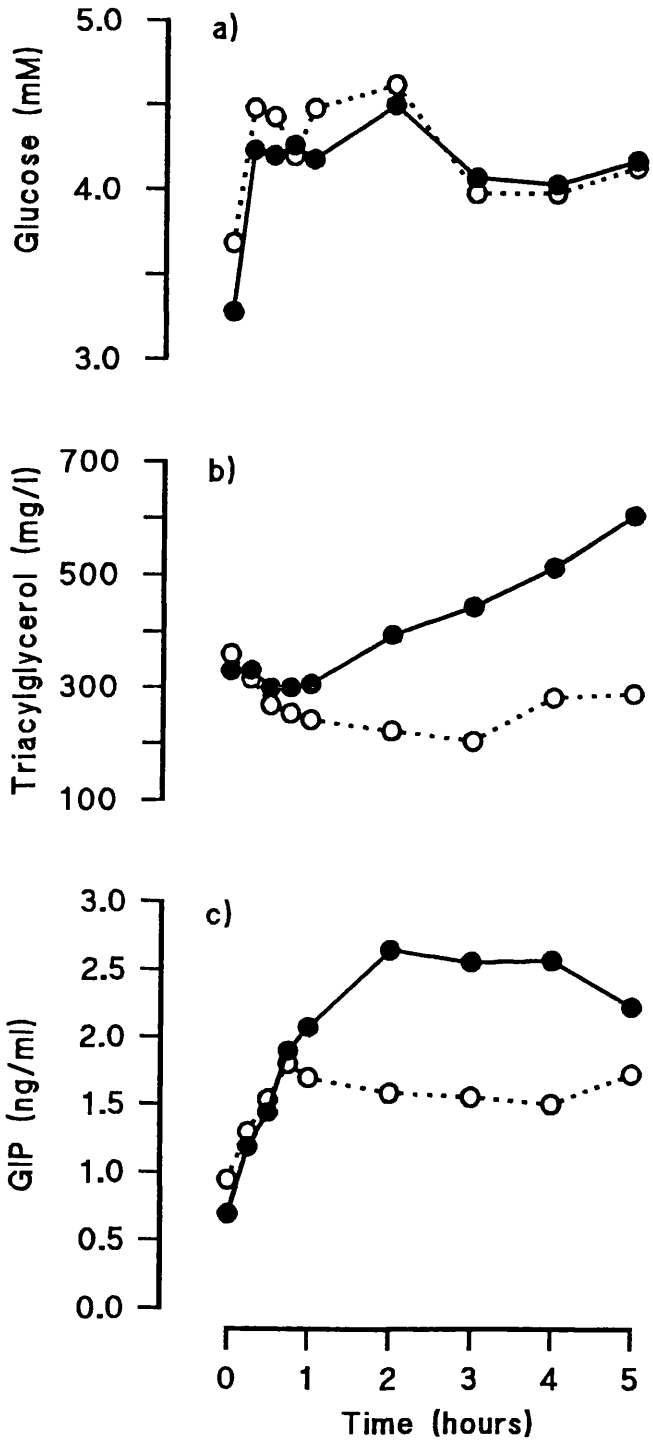



**Figure 3.8.** Plasma concentrations of (a) glucose, (b) triacylglycerol and (c) GIP in ruminant goat kids given milk (●—●) or skimmed milk (○---○). Data are means for 12 animals. S.E.D. values for (a), (b), (c) within feeds are 0.26 mM, 22.6 mg/l and 0.24 ng/ml respectively, and those between feeds are 0.35 mM, 47.2 mg/l and 0.45 ng/ml.

significant decrease ($P<0.01$) in plasma triacylglycerol concentration within 30 minutes and remained below basal for the duration of sampling ($P<0.001$). Plasma GIP concentration was significantly increased ($P<0.05$) within 15 minutes of skimmed milk ingestion, and remained so throughout the sampling period ($P<0.01$).

**Response to cream**

Plasma concentrations of glucose, triacylglycerol and GIP in 5 goat kids given milk and, on a separate occasion, milk fat as cream are shown in Figure 3.9. (a, b, and c, respectively). When cream was ingested, plasma glucose concentrations remained at basal (Figure 3.9.a), whereas triacylglycerol concentrations were increased ($P<0.01$) within 2 hours, and remained elevated throughout the sampling period ($P<0.05$). Plasma GIP concentration was significantly increased ($P<0.001$) 4 hours after ingestion of cream.

**Response to lactose, glucose, casein, casein with lactose, soya protein isolate with lactose or casein hydrolysate with lactose.**

Plasma GIP concentrations in goat kids after ingestion of lactose, casein, casein with lactose and when no feed was given are shown in Figure 3.10 with, for comparison, responses to milk and skimmed milk in the same animals. In contrast to marked GIP responses to milk and skimmed milk, ingestion of lactose or casein failed to elicit GIP secretion. Consistent with the absence of a response to lactose, GIP concentrations in 3 other animals given glucose were 0.68 ng/ml before feeding and 0.67, 0.65, 0.61, 0.64, 0.58 and 0.48 (S.E.D. = 0.096) ng/ml at sampling times during the subsequent 3 hours. However, consumption of casein with lactose induced a moderate GIP response, with GIP concentrations increased ($P<0.05$) within 30 minutes of feeding and peaking 2 to 3 hours after feeding ($P<0.01$).

Figure 3.11 shows the GIP responses in a further 4 animals after consumption of different protein sources (casein and soya protein

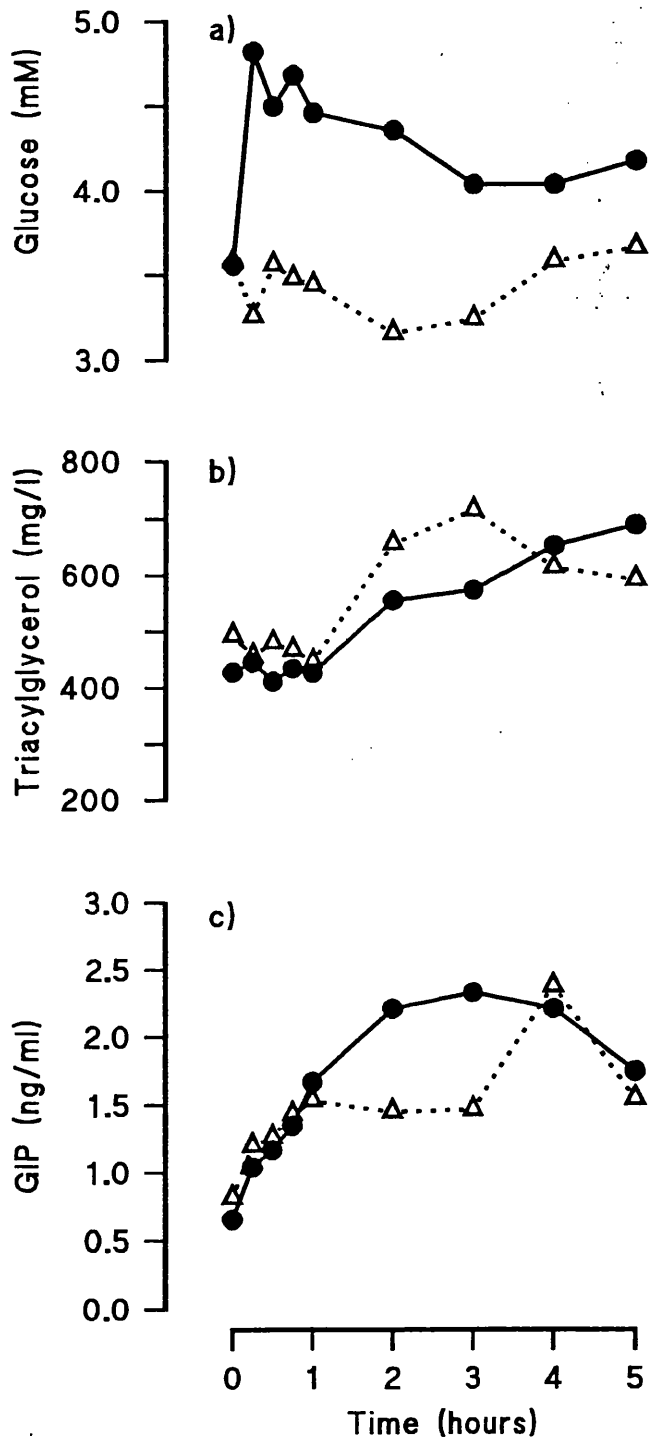



**Figure 3.9.** Plasma concentrations of (a) glucose (b) triacylglycerol and (c) GIP in ruminant goat kids given milk (●—●) or cream (△--△). Data are means from 5 animals. S.E.D. values for (a) (b) and (c) within feeds are 0.23 mM, 48.6 mg/l and 0.39 ng/ml respectively, and those between feeds are 0.34 mM, 75.3 mg/l and 0.51 ng/ml.

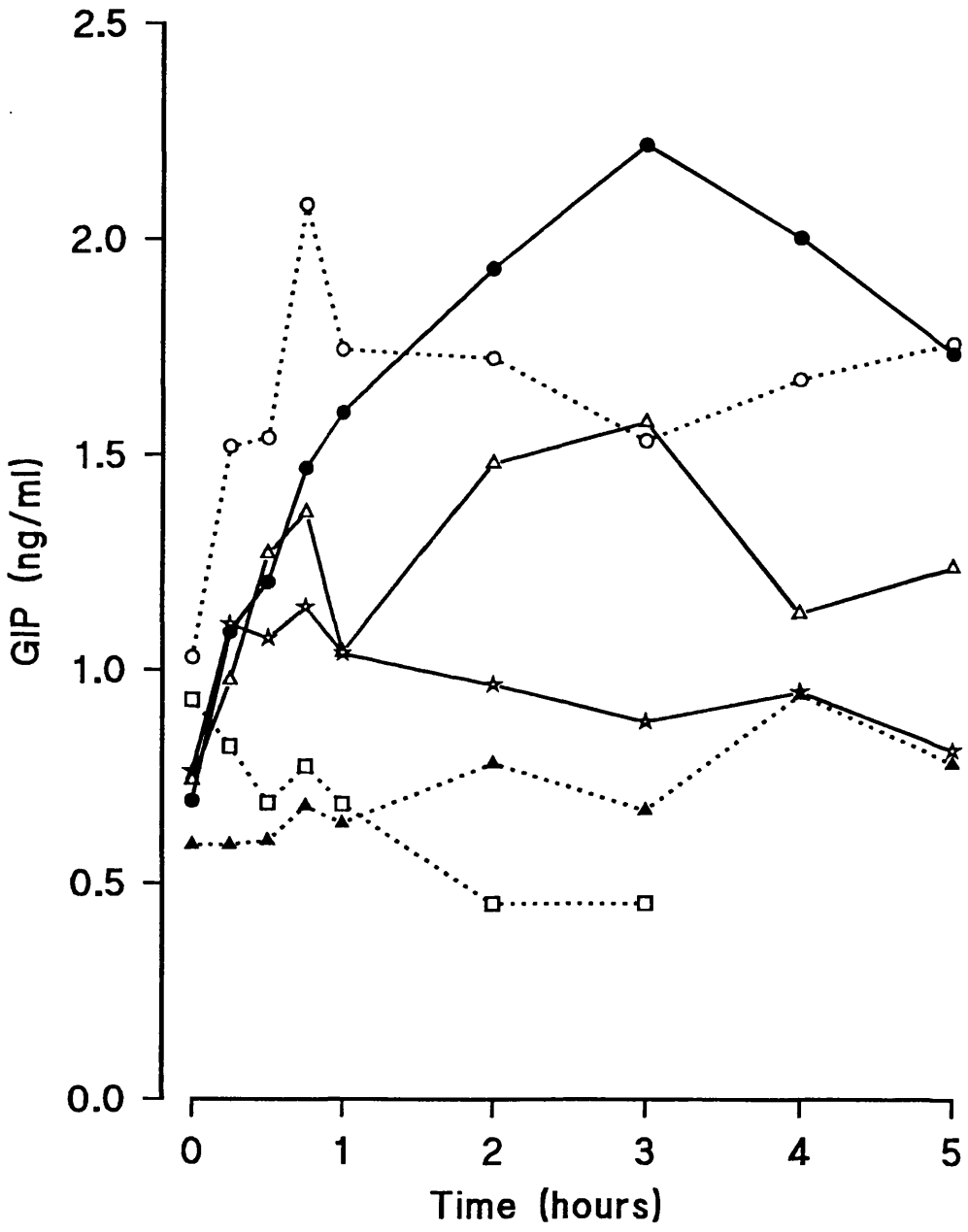



**Figure 3.10.** Plasma concentrations of GIP in ruminant goat kids given milk (●—●), skimmed milk (○···○), casein + lactose (△—△), casein (★—★), lactose (□···□) and no feed (▲···▲). Data are means of 4 animals. S.E.D. within feeds was 0.25 ng/ml, and between feeds 0.44 ng/ml.

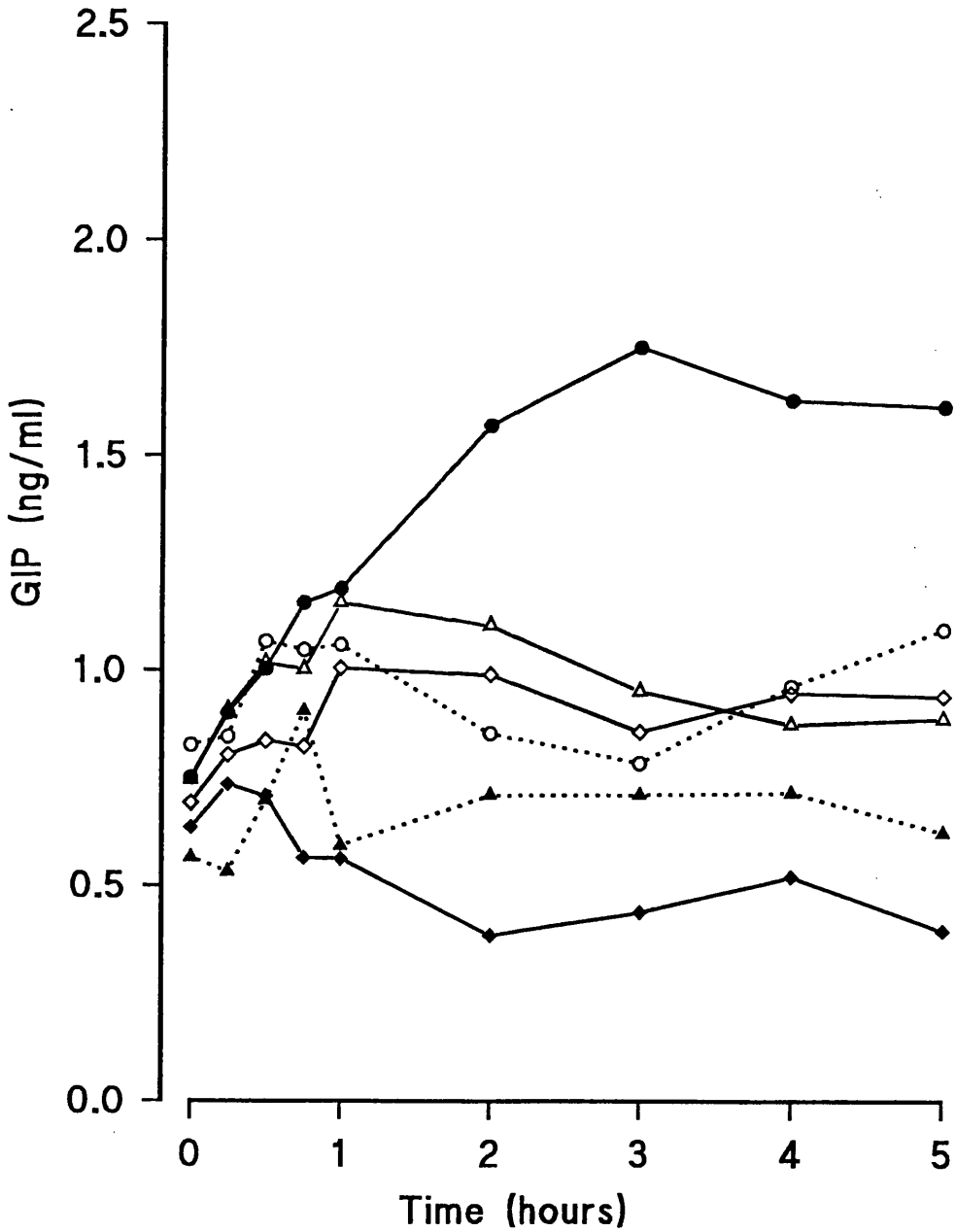



**Figure 3.11.** Plasma concentrations of GIP in ruminant goat kids given milk (●—●), skimmed milk (○····○), casein + lactose (△—△), soya protein isolate + lactose (◇—◇), casein hydrolysate + lactose (◆—◆) or, no feed (▲···▲). Data are means of 4 animals. S.E.D. within feeds was 0.17 ng/ml, and between feeds 0.34 ng/ml.

isolate) and of casein in the form of its constituent amino acids (casein hydrolysate), all given with lactose. For reasons that are unclear, the response to milk in these animals was relatively small and there were no significant changes in GIP concentration after consumption of skimmed milk. In these animals changes in GIP concentrations after consumption of casein with lactose were small and were significant only at 1 and 2 hours after feeding. Consumption of soya protein isolate with lactose did not significantly affect GIP concentrations; as when no feed was given there was a tendency for GIP concentrations to rise slightly after feeding but otherwise to remain close to prefeed values. There was no indication of a positive response to casein hydrolysate with lactose, with GIP concentrations tending to fall during the sampling period.

**Response to concentrates**

Plasma acetate concentrations were monitored, as an index of rumen fermentation, at hourly intervals after feeding in 4 of the 9 animals given concentrates and hay. Whereas milk consumption had no effect on plasma acetate concentration, acetate was significantly increased ($P < 0.001$) above prefeed levels 1 hour after consumption of concentrates and hay and remained so for the 5-hour sampling period: for milk, mean prefeed acetate concentration was 0.38 mM and hourly after feeding was 0.35, 0.35, 0.37, 0.31, and 0.34 mM; corresponding values for concentrates were 0.44, 1.05, 1.13, 1.16, 1.04 and 1.02 mM (S.E.D. between feeds 0.110, and within feeds 0.063 mM).

Mean GIP concentrations, for 9 animals, before and after consumption of concentrates and hay were 0.73, 0.76, 1.11, 1.06, 1.17, and 1.25 ( S.E.D. =0.189) ng/ml, showing a significant increase 4 and 5 hours after feeding ($P < 0.05$ and $P < 0.01$ respectively).

**Comparison of GIP responses to different feeds**

When the results for all animals were combined for each test meal, overall response expressed as the integrated mean change in GIP

**Table 3.2.** Integrated mean changes in the concentrations of GIP, glucose and triacylglycerol over a five hour period in ruminant goat kids fed test meals.

| | milk | skimmed milk | cream | casein + lactose | no feed | SED (range) |
|---|---|---|---|---|---|---|
| GIP (ng/ml) | ** $0.65^{a}$ | $0.29^{b}$ | $0.44^{ab}$ | $0.25^{b}$ | $0.23^{b}$ | 0.13 - 0.09 |
| Glucose (mM) | ** $0.37^{a}$ | $0.18^{b}$ | $0.05^{b}$ | $0.11^{b}$ | $0.02^{b}$ | 0.12 - 0.08 |
| Triacylglycerol (mg/l) | *** $94.3^{a}$ | $-31.4^{c}$ | $41.1^{b}$ | N.D. | $-18.4^{a}$ | 21.1 - 13.1 |
| n | 12 | 12 | 5 | 8 | 7 | |

N.D. = not determined.

Mean changes which are significantly different from zero are ** $P<0.01$, *** $P<0.001$.

$^{abc}$Means having different superscripts are significantly different ($P<0.05$).

concentration was significant only after milk consumption (Table 3.2). The integrated mean change in GIP after cream consumption did not differ significantly from that after milk, but though cream ingestion tended to give an overall response this was not greater ($P > 0.05$) than when other feeds, or indeed no feed, were given. Likewise, only milk was associated with significant integrated mean increases in plasma glucose and triacylglycerol concentrations.

## DISCUSSION

Plasma levels of acetate increased markedly during the postprandial period after ingestion of concentrates indicating that, as intended, these animals had developed a degree of rumen function. Plasma acetate concentrations remained constant after milk ingestion, consistent with the flow of liquid feeds through the oesophageal groove directly to the small intestine. As further evidence of this, plasma glucose and triacylglycerol concentrations increased rapidly after milk was given.

There were marked GIP responses after milk ingestion but these were more variable than those observed in the pre-ruminant goat. The reason for this variability is not clear, but it was possibly a reflection of variations in the contribution of outflow of digesta from the rumen to nutrients passing to the small intestine in these ruminating animals and of any effects this flow may have had on the rates of passage and of absorption of the test meal in the small intestine. Also, the presence of digesta in the abomasum may have modified the formation of milk clot there. Again in contrast to the pre-ruminant goat kids, there was a significant GIP response after ingestion of skimmed milk in some, though not all, of the ruminant kids. However, in common with responses in pre-ruminant goat kids, fat remained a potent GIP secretagogue after the development of rumen function, whereas ingestion of glucose had no effect on GIP release. Therefore, the response to

skimmed milk may have been attributable to fat absorption, but the amounts of fat in this test meal were very small; products of protein digestion may have contributed to the stimulation of GIP release.

In view of indications that constituents of skimmed milk were able to induce GIP secretion, casein and lactose were given individually, but alone had no effect on GIP release. However, there was a GIP response after ingestion of casein with lactose suggesting possible associative effects between these nutrients, perhaps during digestion or metabolism within the K cell. Though again this response was variable it was greater in those animals showing a response to skimmed milk. Ingestion of soya protein isolated from soyabean meal, the protein source commonly included in diets for adult ruminants, had no effect on GIP release. However, it must be noted that the animals in which this meal was tested were those that showed a relatively small response to milk and no response to skimmed milk, so perhaps a GIP response to protein might not have been expected. Further investigation of the GIP response to protein ingestion demonstrated that casein hydrolysate with lactose failed to elicit a GIP response. This indicated the ability of protein to induce a response was not dependent on the absorption of individual amino acids, but rather on absorption of peptides released during protein digestion. This contrasts with the GIP responses to amino acids (Thomas *et al.*, 1976), but not to intact protein (Cleator and Gourlay, 1975), reported in simple-stomached animals. However, it is known that amino acids are absorbed more rapidly when in the form of di- and tri-peptides rather than free amino acids (Adibi and Kim, 1981). Therefore, amino acid uptake may have been greater when casein rather than casein hydrolysate was given, more so if flow of casein hydrolysate which would not have clotted in the abomasum, through the small intestine was so rapid as to reduce amino acid absorption. Unfortunately, it was not possible to measure changes in plasma amino acids after feeding in the experiment reported here.

Encouragingly, the increases in GIP concentration after

concentrate consumption demonstrated that the ability to evoke GIP release was not confined to milk and milk constituents, and suggested that fat, and possibly protein, of dietary and/ or microbial origin, may elicit GIP release in the ruminating animal.

# CHAPTER 4

## Studies of GIP secretion in adult sheep

## EXPERIMENT 4.1 CHANGES IN CIRCULATING CONCENTRATIONS OF GIP IN RESPONSE TO FEEDING IN FASTED SHEEP

### INTRODUCTION

The results of experiments in Chapter 3 demonstrated marked changes in GIP concentration in response to milk consumption in pre-ruminants (Experiment 3.2), and that the response is retained after the development of rumen function in the goat kid (Experiment 3.3), with fat absorption being the predominant stimulus for GIP secretion. However, the young ruminant animals used in Experiment 3.3 were consuming milk as part of their diet and, as this would by-pass the rumen to be digested in the small intestine, these animals were not entirely representative of adult ruminants.

As discussed in Chapter 1, patterns of digestion and nutrient absorption in the adult ruminant differ markedly from those in pre-ruminant and simple-stomached animals. Notably, because of extensive fermentation of dietary carbohydrate in the rumen, relatively small amounts of glucose are absorbed from the small intestine (Leng, 1970; Bergman, 1975). Also, fat constitutes only 3 to 5 % of the typical diet for ruminants and long-chain fatty acids make a relatively small contribution to total nutrient supply compared with the situation in pre-ruminants and, for example, man (Byers and Schelling, 1988). As a result of microbial activity in the rumen, fat reaches the small intestine in the form of unesterified long-chain fatty acids, predominantly 18:0, rather than as triacylglycerol. Also, the large volume of the rumen buffers the flow of digesta to the small intestine. It may be anticipated therefore that in adult ruminants, the nutrients affecting GIP secretion, and possibly the site of secretion, will differ from those in simple-stomached animals. The following experiment was conducted with the aim of identifying both the site and effectors of GIP secretion

in the adult ruminant by relating the changes in plasma GIP concentration which occur in sheep after refeeding following a 48-hour fast to the changes in plasma concentrations of various metabolites indicative of nutrient absorption.

## EXPERIMENTAL

### Animals and their management

Six Finn-Dorset Horn cross-bred male sheep aged 8-12 months were used in the experiment. Animals were fed 600 g concentrates/day (goat mix 1, Edinburgh School of Agriculture: crude protein 165 g/kg, metabolizable energy 12.5 MJ/kg) in two equal meals at 08.00 and 16.00 hours. Hay and water were available *ad libitum*.

### Experimental procedure

A polyethylene catheter (Internal Diameter 0.80 mm; Dural Plastics, Dural, Australia) was placed in a jugular vein on the day before the start of the experiment and blood samples were taken through this catheter.

Sheep were starved for 48 hours prior to the experiment. On the day of the experiment, animals were fed 300 g concentrates at 09.00, 12.00, and 15.00 hours and given free access to hay. Blood samples (5ml) were withdrawn into heparinized syringes prior to nutrient ingestion and at 0, 15, 30, and 60 minutes, and then hourly, for a total of 7 hours after the initial feed. Blood was mixed immediately with 1000 KIU aprotinin/ml, centrifuged at 1300 *g* for 15 minutes at 4°C, plasma removed and stored at -20°C until analysed for GIP, glucose, triacylglycerol, $\beta$-hydroxybutyrate and acetate, as described in Chapter 2.

### Statistical analysis

All data are given as means with S.E.D. Statistical analysis was

by analysis of variance and significance was determined by variance ratios and t-test. Paired t-tests were also used.

## RESULTS

### Plasma GIP concentrations

In adult sheep, plasma GIP concentrations increased from basal levels of about 0.25 ng/ml at time = 0 to peak values of approximately 0.55 ng/ml after refeeding (Figure 4.1). The rise was statistically significant ($P < 0.05$) 2 hours after refeeding following an apparent lag phase of about 1 hour during which no changes were apparent. Peak concentrations were reached by about 3-4 hours.

### Plasma acetate and β-hydroxybutyrate concentrations

Concentrations of acetate and β-hydroxybutyrate increased rapidly following feeding (Figure 4.2). The plasma concentrations of acetate were significantly higher ($P < 0.01$) than basal levels by 1 hour after refeeding (Figure 4.2.a). The concentration of β-hydroxybutyrate also increased and was significantly higher ($P < 0.05$) than basal 30 minutes after feeding (Figure 4.2.b). Peak concentrations were reached about 4 hours after refeeding. The change in concentration of plasma β-hydroxybutyrate correlated significantly ($r=0.937$; $P < 0.001$) with that of acetate.

### Plasma triacylglycerol and glucose concentrations

The concentration of triacylglycerol increased after feeding but showed a lag phase of 2 hours before the increases in concentration were apparent (Figure 4.3.a). After this, concentrations increased slowly between 3 to 7 hours after feeding, being significantly higher ($P < 0.05$) than basal 3 hours after feeding. The increase in plasma glucose concentration (Figure 4.3.b) was small and was not statistically significant.

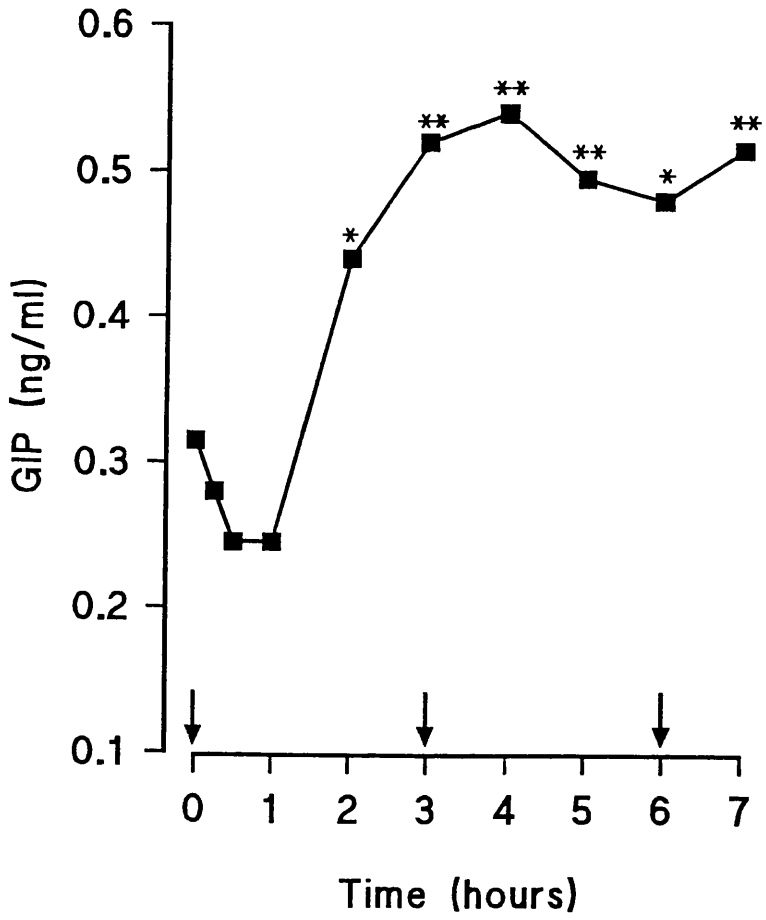



**Figure 4.1.** Changes in the concentration of GIP in plasma of sheep before and after feeding. Sheep were fed at 09.00, 12.00, 15.00 hours as indicated by arrows. Data are means from six animals, with S.E.D. of 0.057 ng/ml. * $P<0.05$, ** $P<0.01$ compared with values before feeding.

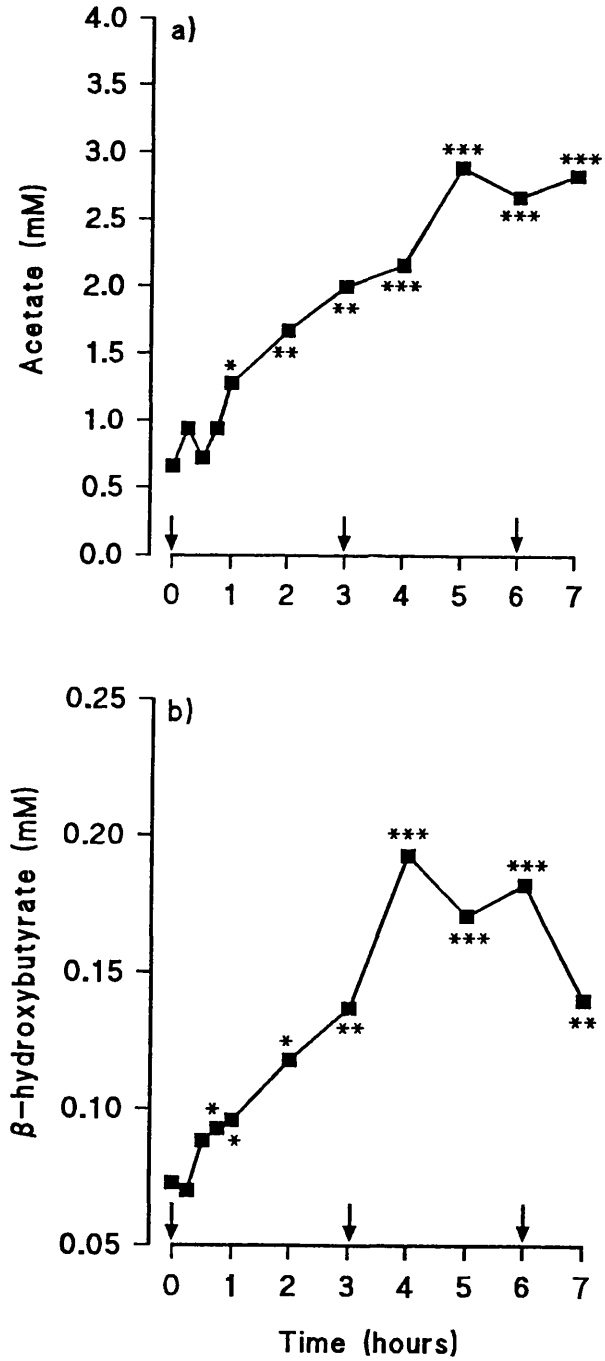



**Figure 4.2.** Changes in the concentrations of (a) acetate and (b) β-hydroxybutyrate in plasma of sheep before and after feeding. Sheep were fed at 09.00, 12.00, 15.00 hours as indicated by arrows. Data are means from six animals, with S.E.D. of 0.403 mM for acetate and 0.022 mM for β-hydroxybutyrate. *P<0.05, **P<0.01, ***P<0.001 compared with values before feeding.

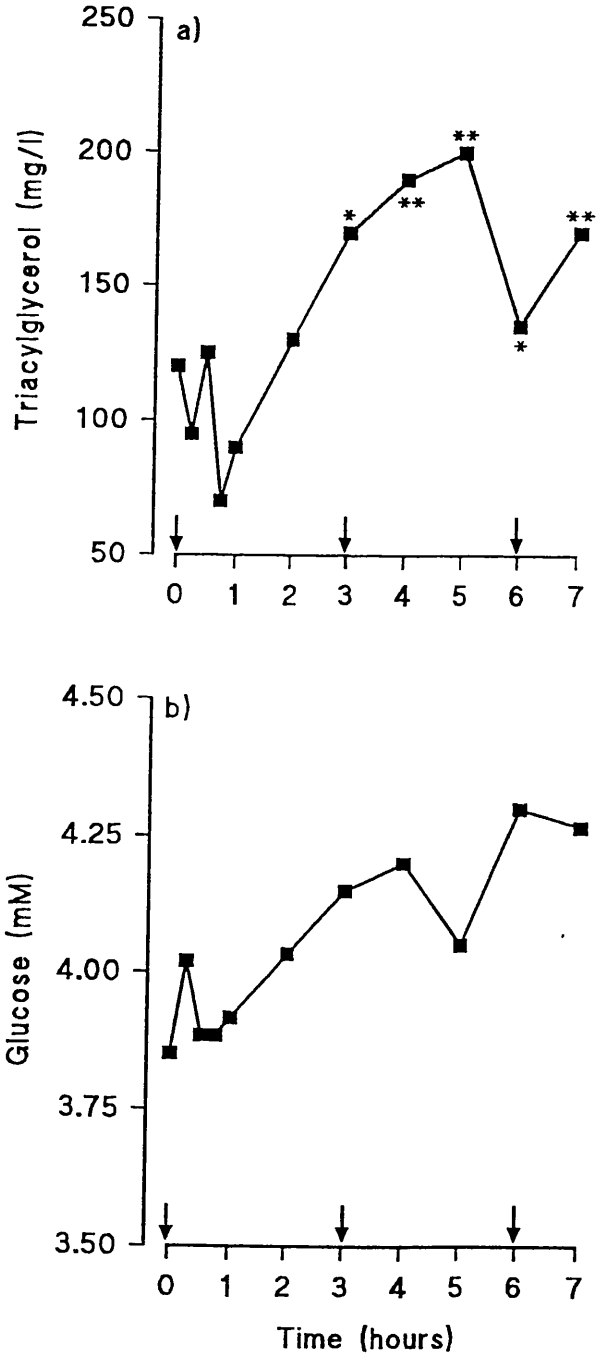



**Figure 4.3.** Changes in the concentrations of (a) triacylglycerol and (b) glucose in plasma of sheep before and after feeding. Sheep were fed at 09.00, 12.00, 15.00 hours as indicated by arrows. Data are means from the six animals, with S.E.D. of 24 mg/l for triacylglycerol and 0.23 mM for glucose. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with values before feeding.

## DISCUSSION

Plasma GIP concentrations in the adult sheep increased after nutrient ingestion. The response was significant 2 hours after feeding, was maximal by 3-4 hours but was less than that in young ruminant goats after milk ingestion in Experiment 3.3.

Increases in plasma acetate concentrations can be taken as an index of acetate absorption from the rumen. On the basis of changes observed in this study, acetate absorption precedes the increase in plasma GIP concentrations by at least 1 hour and so is unlikely to be a stimulus for GIP secretion. Similarly, increases in plasma $\beta$-hydroxybutyrate rapidly follow feeding and precede the changes in GIP concentrations. $\beta$-hydroxybutyrate is produced in the rumen epithelial cells by metabolism of butyrate (Fahey and Berger, 1988), so changes in its concentration in plasma probably reflect butyrate absorption. No attempt was made to relate propionate absorption to changes in GIP concentrations, but previous workers have shown that this VFA is also absorbed rapidly and concentrations in portal blood have been reported to be high within 1 hour after feeding in sheep (Thompson, Bassett and Bell, 1978). In addition, absorption of both propionate and butyrate has been shown to be faster than that of acetate (Merchen, 1988) so that it is unlikely that the absorption of any of the VFA was a stimulus for GIP secretion in these sheep. The likelihood that none of these nutrients absorbed from the rumen elicit GIP secretion is consistent with the rumen being lined with non-glandular stratified squamous epithelium (Stevens, 1988) and the reported absence of K cells in the rumen (Bunnett and Harrison, 1986).

Very little glucose is absorbed in ruminants, as was evident from the lack of any significant post-prandial change in plasma glucose concentrations in this study. Thus it is unlikely that glucose had any role in eliciting the GIP secretion seen here in adult sheep. Moreover, the results of experiments in Chapter 3 indicate that even if glucose were to

be absorbed from the small intestine this would not have elicited GIP secretion.

Saturated, long-chain free fatty acids of dietary and microbial origin are absorbed from the small intestine, re-esterified within the intestinal cell and finally secreted into plasma via the lymphatic system (Leat and Harrison, 1975). Leat and Harrison (1974) demonstrated a delay of about 45 minutes between the introduction of $^{3}$H-palmitic acid into the duodenum of sheep and the appearance of radioactivity in lymph. Hence a delay of about 1 hour between the absorption of fatty acids in the small intestine and the appearance of triacylglycerol in plasma would be predicted. This corresponds closely to the delay observed in the present investigations between the increases in the plasma concentrations of GIP and those of triacylglycerol, increases in GIP concentrations preceding those of triacylglycerol by about 1 hour. Thus the long-chain free fatty acids appear to be likely candidates as secretagogues whose absorption elicit GIP secretion. This is consistent with the observations in young ruminant goat kids after the ingestion of milk and milk constituents.

The absorption of amino acids from the small intestine, although not as effective as fat, has been shown to elicit GIP secretion in mice (Flatt *et al.*, 1984; Flatt, Kwasowski, Howland and Bailey, 1991). It remains possible that amino acids could play a role in eliciting GIP secretion in ruminants.

## EXPERIMENT 4.2. GIP CONCENTRATIONS DURING THE DEVELOPMENT OF OBESITY IN SHEEP

### INTRODUCTION

Results of Experiment 4.1 demonstrated changes in plasma GIP concentration during nutrient absorption in adult sheep. As described in Chapter 1, GIP release in simple-stomached animals is dose-dependent (Martin, Sirinek, Crockett, O'Dorisio, Mazzaferri, Thompson and Cataland, 1975; Schlesser, Ebert and Creutzfeldt, 1986) and modified by preceding level of energy intake (Reiser *et al.*, 1980; Ponter *et al.*, 1991). Furthermore, humans (Elahi, Anderson, Muller, Tobin, Brown and Andres, 1984) exhibit hyperinsulinaemia, hyperglycaemia and insulin resistance during obesity and it has been suggested that exaggerated GIP secretion and overactivity of the enteroinsular axis may be involved in this, since obese sheep exhibit the same features (McCann, Bergman and Reimars, 1989) a similar role for GIP may operate during obesity in ruminants.

To investigate the involvement of GIP in the development of obesity in sheep, plasma samples were obtained from a study involving the nutritional manipulation of fat deposition in sheep conducted by J.McCann (College of Veterinary Medicine, Oklahoma State University, U.S.A.). Plasma GIP concentrations were measured at time points for a 71-week period in a group of lean animals and, in a second group of animals, during the dynamic and static phases of obesity. GIP concentrations were related to the changes in liveweight and insulin concentrations measured by McCann.

### EXPERIMENTAL

Experimental details were similar to those of a previous study by McCann, Bergman and Beerman (1992). For several weeks before the

first day of the experiment, the sheep were group-fed, receiving sufficient concentrates and hay to provide the calculated requirements for sheep weighing about 45 kg (McCann, Bergman and Beerman, 1992).

Sheep were then assigned randomly to obese (n=5) and lean (n=5) groups. The animals were fed on a pelleted hay-grain diet at maintenance (lean) or fed the same diet *ad libitum* (obese) for a 71-week period. The first day of ad libitum feeding was considered day 0 of the experiment. Body weights and plasma insulin concentrations were measured by J.McCann throughout most of the experiment. Blood samples were taken postprandially from a jugular vein by venepuncture into tubes (Vacutainer; Becton-Dickinson Ltd, Wembley, U.K.) and mixed immediately with heparin (10 units/ml) and benzamidine solution (200 mg/ml), then transported frozen on dry ice to Hannah Research Institute. Plasma GIP concentrations were measured in samples taken on weeks 1, 4, 10, 20, 30, 40, 50 and 71, as described in Chapter 2. Insulin concentrations in corresponding samples were determined by McCann.

### Statistical analysis

Differences in postprandial GIP concentrations between the groups of lean and of obese sheep were assessed for statistical significance using Students t-test.

## RESULTS

### Body weight and plasma insulin concentrations

The mean live weight in the group of lean sheep remained unchanged at approximately 45 kg throughout the experiment (Figure 4.4.a), whereas the weight in the group of obese sheep doubled when they were allowed *ad libitum* intake of nutrients throughout the experimental period. Body weights in the obese sheep reached a plateau at approximately 95 kg around week 40.

In the group of lean sheep, postprandial concentrations of insulin

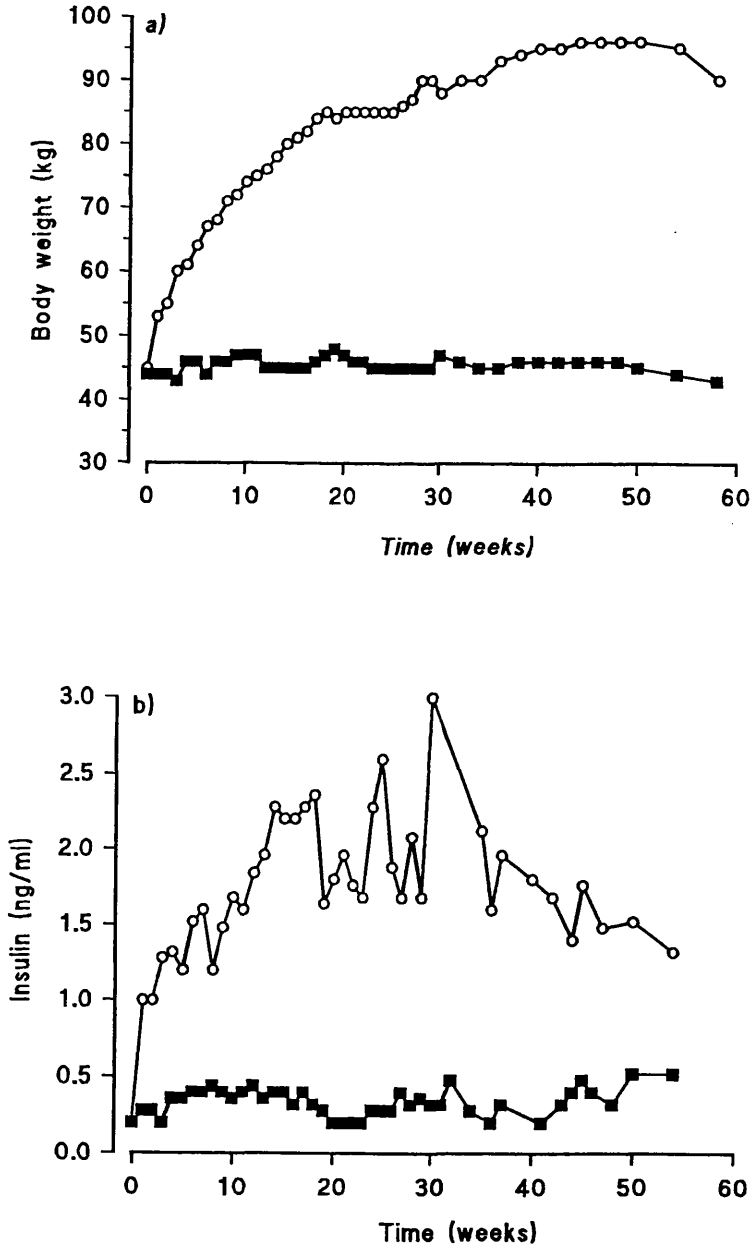



**Figure 4.4.** (a) Bodyweight and (b) plasma insulin concentration in sheep allowed *ad libitum* intake (obese, ○—○) and those fed at the maintenance level (lean, ■—■).

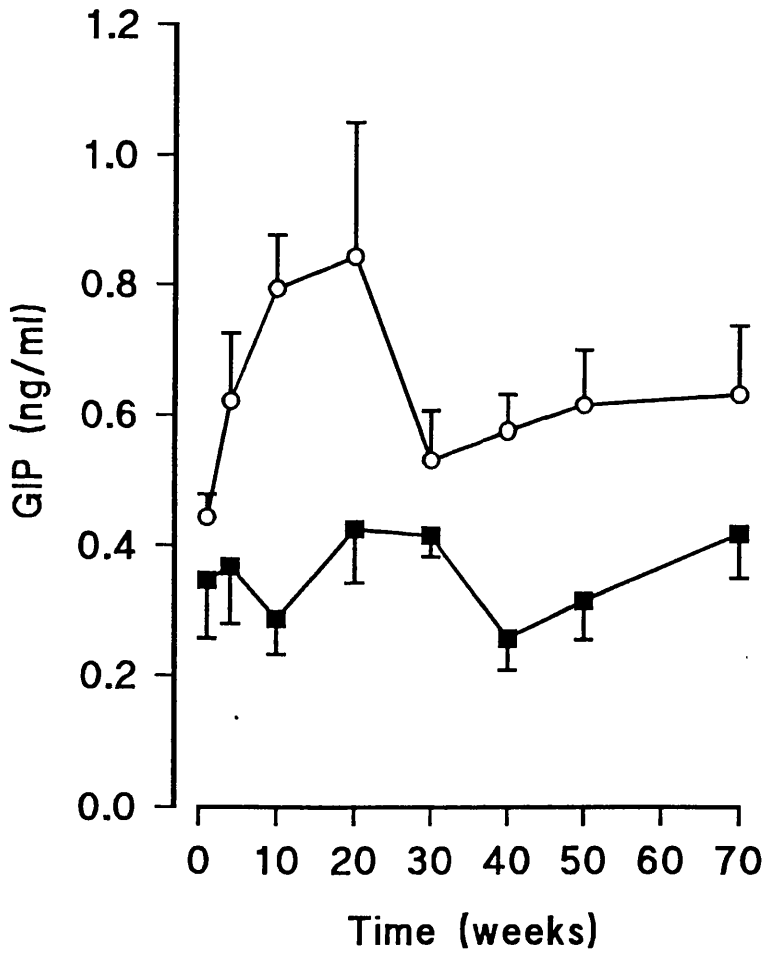



**Figure 4.5.** Plasma GIP concentration in sheep allowed *ad libitum* intake (obese, o—o) and those fed at the maintenance level (lean, ■—■). Data are means, with S.E.M., of 5 animals.

remained unchanged throughout the period of sampling (Figure 4.4.b). Postprandial plasma concentrations of insulin in the group of obese sheep increased steadily until approximately week 30 and were consistently higher than levels in the lean sheep after 3 weeks of the experiment. Maximum insulin levels in the obese sheep were achieved by week 30, then declined throughout the remaining sampling period.

**Plasma GIP concentrations**

In the group of lean sheep, postprandial GIP concentrations in the samples taken throughout the experiment were approximately 0.4 ng/ml (Figure 4.5). In the obese sheep, plasma GIP concentrations postprandially were significantly ($p < 0.05$) increased from approximately 0.45 ng/ml at week 0 within the first 5 weeks of *ad libitum* feeding, and reached maximum concentrations of approximately 0.85 ng/ml by week 20. Plasma GIP concentrations then declined and remained relatively constant at approximately 0.6 ng/ml until the end of the experimental period. In obese sheep, plasma GIP concentrations were consistently higher than those in lean sheep ($P < 0.05$).

## DISCUSSION

It appears that plasma GIP concentrations observed in sheep are directly related to the level of dietary intake. When sheep were fed a maintenance diet GIP levels did not differ between the time points during the experiment, whereas in the obese sheep, during the dynamic phase of obesity (weeks 0-20) hyperphagia, which can be inferred from changes in liveweight, was associated with exaggerated GIP secretion. Furthermore, during the static phase of obesity (weeks 30-70), when presumably obese sheep had a lower dietary intake than during the dynamic phase, plasma GIP concentrations were reduced. GIP levels remained significantly greater than those of lean sheep; this difference is probably attributable to moderately higher intakes in the obese sheep;

in line with the differences in live weight of the two groups at this stage of the experiment these animals would have had a higher maintenance requirement than the lean animals. These observations were consistent with findings in pigs (Ponter *et al.*, 1991) and humans (Morgan *et al.*, 1988 a), that GIP secretion could be enhanced by increasing the level of dietary intake. The increase in postprandial GIP concentration in the obese sheep coincided with the increase in serum insulin levels. This indicated that GIP could be involved in the regulation of insulin secretion and/ or, since liveweights were increasing at the same time, in tissue deposition. Further studies could investigate the effect of dietary intake levels on GIP secretion in different metabolic states, for example during lactation, which are associated also with hyperphagia.

## EXPERIMENT 4.3. COMPARISON OF THE CHANGES IN CIRCULATING CONCENTRATIONS OF GIP IN RESPONSE TO FEEDING IN LACTATING AND NON-LACTATING SHEEP

### INTRODUCTION

The results of Experiment 4.1 demonstrated that GIP is secreted in response to nutrient ingestion in adult sheep. Experiment 4.2 showed that during the development of obesity, excessive nutrient intake was associated with greater GIP release. During lactation dietary intake increases substantially and this could also be associated with increased GIP secretion. Thus, it is possible that GIP could play a role in the regulation of nutrient utilization during lactation. However, if GIP has effects in ruminants similar to the insulin-mediated and direct, insulin-like effects in simple-stomached animals, GIP would seem more likely to favour nutrient partitioning towards body tissue. The aim of this experiment was to determine whether GIP concentrations, and responses to nutrient intake are in fact greater during lactation.

### EXPERIMENTAL

#### Animals and their management

Lactating (day 18-22 of lactation; n=13) and non-lactating (n=13) Finn-Dorset Horn cross-bred sheep aged 3-4 years were used in the experiment. In the period before the experiment the normal feed intake for the non-lactating sheep was 500 g concentrates/day, whereas the lactating sheep ingested 1000 g concentrates/day. Meals of equal size were given at 08.00 and 16.00 h and hay and water were available *ad libitum*.

#### Experimental procedure

A polyethylene catheter (Internal Diameter 0.8 mm; Dural

Plastics, Dural, Australia) was placed in the jugular vein on the day before the start of the experiment and blood samples were taken through this catheter. Sheep were starved for 24 hours prior to the experiment. Both lactating and non-lactating sheep on separate days, at least three days apart, were fed one of two levels of feed (250 g or 500 g concentrates, *i.e.*, amounts equivalent to a normal feed for the non-lactating and lactating sheep respectively). On the day of the experiment animals were fed at 09.00 hours and given free access to hay. Two pre-feed blood samples (5 ml) were taken and further samples were taken at 30-minute intervals for a total of 7 hours after feeding. Blood was mixed immediately with heparin (10 units/ml) and aprotinin (1000 KIU/ml; Sigma, Poole, U.K.), centrifuged at 1300 *g* for 15 minutes at 4°C, and plasma removed and stored at -20°C until analysed for GIP, glucose and triacylglycerol as described in Chapter 2.

### Statistics

All data are given as means with S.E.D. Statistical analysis was by analysis of variance and significance was determined by variance ratios and t-tests.

## RESULTS

### Plasma glucose response

Plasma glucose levels in the fasting state were significantly greater ($P < 0.05$) in the non-lactating than in the lactating sheep (Figure 4.6). In non-lactating sheep ingestion of their normal meal of concentrates (250 g) increased plasma glucose levels ($P < 0.05$) within 2.5 hours. Glucose concentrations remained elevated above basal for the duration of the sampling period. After ingestion of 500 g concentrates, the higher than normal feed level, plasma glucose levels increased significantly ($P < 0.05$) within 2 hours, reached a maximum level of approximately 3.8 mM by 3 hours and remained significantly elevated

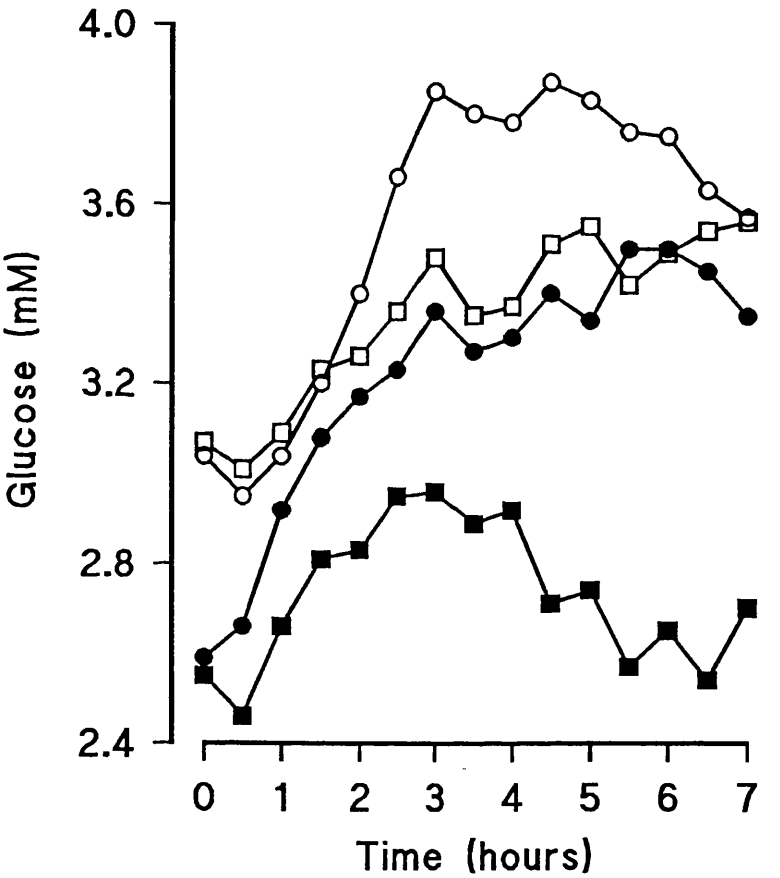



**Figure 4.6.** Changes in plasma glucose concentrations in lactating and non-lactating sheep after ingestion of either 250 g or 500 g concentrates. Sheep were fed at time = 0. Lactating sheep with concentrates at 250 g (■—■) or 500 g (●—●). Non-lactating sheep with concentrates at 250 g (□—□) or 500 g (○—○). Data are means of 13 animals, with S.E.D., within and between feed levels respectively, of 0.14 mM and 0.16 mM.

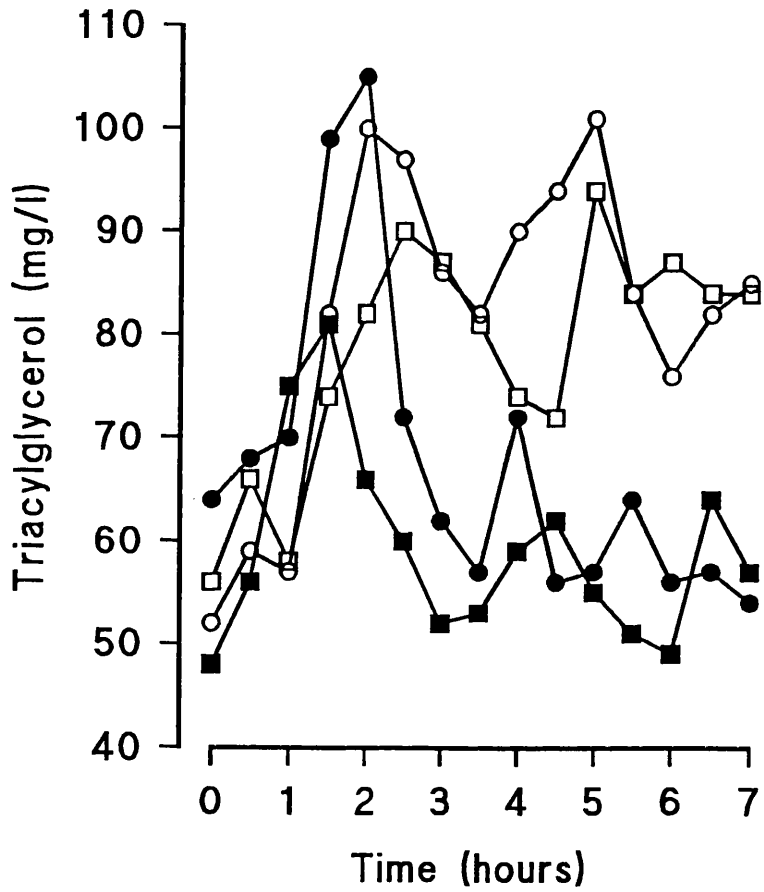



**Figure 4.7.** Changes in plasma triacylglycerol concentrations in lactating and non-lactating sheep after ingestion of either 250 g or 500 g concentrates. Sheep were fed at time = 0. Lactating sheep with concentrates at 250 g (■—■) or 500 g (●—●). Non-lactating sheep with concentrates at 250 g (□—□) or 500 g (○—○). Data are means of 13 animals, with S.E.D., within feeds and between feed levels respectively, of 9.8 mM and 9.5 mM.

($P < 0.001$) above basal. Plasma glucose levels after the higher feed level were generally greater ($P < 0.05$) than after the lower level from 3 to 5.5 hours after feeding.

In lactating sheep, after ingestion of half (250 g) of the amount of feed they normally consumed, plasma glucose levels increased significantly ($P < 0.05$) from a basal concentration of 2.5 mM to 3.0 mM within 2.5 hours, remained at this level for a further 1.5 hours, then declined to the basal concentration. After ingestion of the normal feed level (500 g), plasma glucose levels increased markedly ($P < 0.05$) within 1 hour, and continued to increase, remaining elevated ($P < 0.001$) at approximately 3.4 mM from 3 hours. Thereafter, glucose concentrations were significantly greater ($P < 0.05$) than those observed when 250 g concentrates were given.

Blood glucose concentrations were comparable ($P > 0.05$) in lactating and non-lactating sheep from 1 hour after feeding, and remained so throughout, when their customary level of feed was given.

**Plasma triacylglycerol response**

As shown in Figure 4.7, in non-lactating sheep, ingestion of 250 g concentrates increased the plasma triacylglycerol concentration ($P < 0.01$) within 2.5 hours from approximately 55 to 90 mg/l. The concentration of plasma triacylglycerol remained elevated ($P < 0.01$) for most of the sampling period. After ingestion of 500 g concentrates, plasma triacylglycerol levels were generally comparable ($P > 0.05$) to those for 250 g concentrates throughout the sampling period.

In lactating sheep, after ingestion of 250 g concentrates, plasma triacylglycerol levels increased significantly from approximately 50 to 80 mg/l within 1.5 hours ($P < 0.01$), then declined to the basal of approximately 50 mg/l within 3 hours and remained at this level for the duration of the experiment. After ingestion of 500 g, plasma triacylglycerol levels increased significantly from 65 to 105 mg/l within 1.5 hours ($P < 0.001$), then returned to the basal concentration within

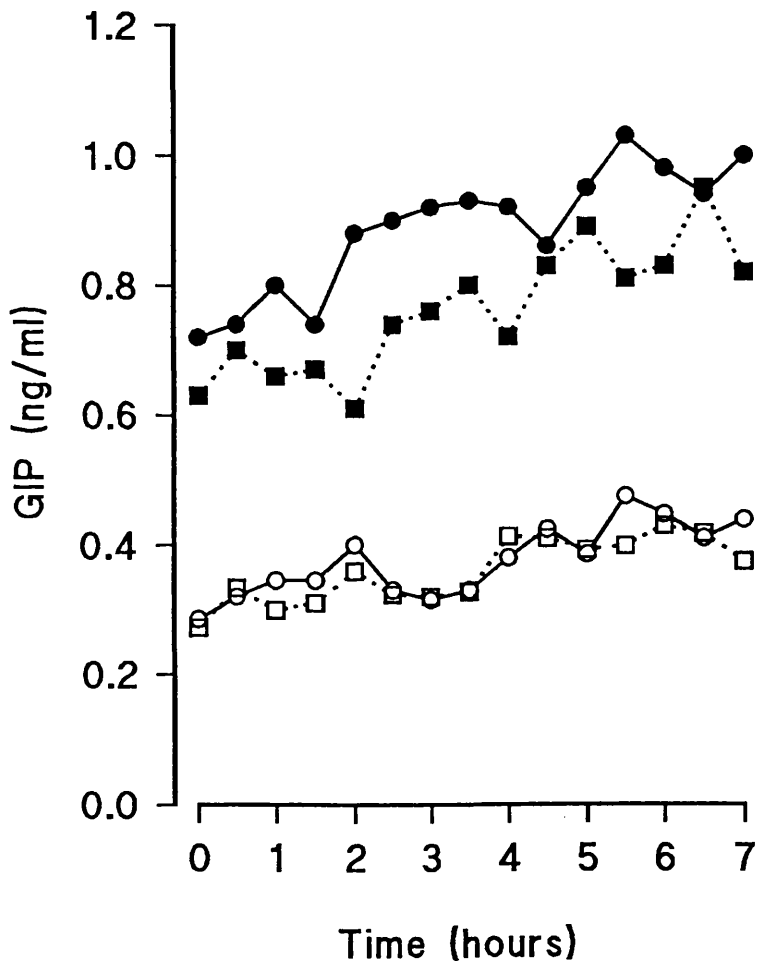



**Figure 4.8.** Changes in plasma GIP concentrations in lactating and non-lactating sheep after ingestion of either 250 g or 500 g concentrates. Sheep were fed at time 0. Lactating sheep with concentrates at 250 g (■···■) or 500 g (●—●). Non-lactating sheep with concentrates at 250 g (□···□) or 500 g (○—○). Data are means of 13 animals, with S.E.D., within and between feed levels respectively, of 0.05 ng/ml and 0.07 ng/ml.

2.5 hours.

**Plasma GIP response**

Plasma GIP levels in the fasting state were significantly greater ($P < 0.01$) in lactating than non-lactating sheep (Figure 4.8). In non-lactating sheep, ingestion of 250 g concentrates led to small increases in plasma GIP levels ($P < 0.05$) within 4 hours. Plasma GIP concentrations generally remained slightly elevated ($P < 0.05$) for the duration of the sampling period. Plasma GIP levels after ingestion of the higher feed level were comparable ($P > 0.05$) to those for the lower feed throughout the sampling period.

In lactating sheep, after ingestion of 250 g concentrates there was a small, but significant ($P < 0.05$) increase in plasma GIP levels within 3 hours. Thereafter GIP concentration continued to increase, reaching a maximum level of 0.96 ng/ml by 6.5 hours. After ingestion of the normal feed level (500 g), plasma GIP levels increased significantly ($P < 0.05$) within 2 hours and remained elevated ($P < 0.001$) for the duration of sampling.

## DISCUSSION

In the fasted state, plasma glucose concentrations were significantly lower in the lactating than in the non-lactating sheep. This difference in plasma glucose levels can be attributed to the glucose requirement of the mammary gland for milk production (Faulkner and Pollock, 1990 b). In lactating sheep, after ingestion of 250 g of concentrates, the increase in blood glucose concentration was significant, but smaller and transient compared with that observed after ingestion of their normal amount of feed. In non-lactating sheep the higher feed level gave greater increases in postprandial glucose concentration than the low level; both levels of feed resulted in consistently greater blood glucose concentrations than those with

comparable feed levels in lactating sheep. These observations were consistent with the metabolic differences between lactating and non-lactating sheep, and confirmed that the differences in level of dietary intake were reflected in nutrient absorption. This is further supported by the apparent differences in postprandial triacylglycerol clearance between lactating and non-lactating animals.

The significant differences in basal GIP concentrations between the lactating and non-lactating sheep could be related to the animals' level of feed intake during the period preceding the experiment. Ponter *et al.* (1991) have shown in pigs that GIP secretion can be enhanced by increasing the level of dietary intake and this was attributed to more releasable GIP in the small intestine. Studies by Fell, Campbell, Mackie and Weekes (1972) have shown that hypertrophy of the gut occurs during lactation. This could result in more releasable GIP, if the density of the K cell population remained constant.

During lactation nutrient partitioning involves the mobilization of fat and protein reserves, increasing gluconeogenesis and directing of nutrients away from tissue deposition to favour milk production. In ruminants, it is known that insulin, glucagon and growth hormone are all associated with this partitioning of nutrients (Bauman and Elliot, 1983). Differences in GIP secretion between lactating and non-lactating sheep supports the involvement of other factors such as gut hormones in milk production. However, on the basis of evidence from simple-stomached animals, GIP would be expected to partition nutrients towards body tissues. This raises questions as to whether the sensitivity of tissues to the effects of GIP are modified during lactation and/ or whether the actions of GIP are different in ruminants.

# CHAPTER 5

## Studies of the actions of GIP in ruminants

## EXPERIMENT 5. EFFECTS OF GIP ON INSULIN SECRETION AND FATTY ACID SYNTHESIS

### INTRODUCTION

Experiments in Chapters 3 and 4 demonstrated that GIP is secreted in young and adult ruminants and that circulating concentrations differ during different physiological states, *i.e.*, obesity and lactation, possibly reflecting altered nutrient intake and absorption. This raises questions as to whether GIP has effects on metabolism in ruminants, and to what extent these effects are similar to those in simple-stomached animals.

In simple-stomached animals GIP is a potent insulin secretagogue, and is one of the main incretin candidates (Creutzfeldt and Ebert, 1985). A possible role for gastrointestinal hormones in the regulation of insulin secretion in ruminants is indicated by the smaller increase in plasma insulin concentrations observed when glucose is given intravenously to starved rather than fed animals (Chaiyabutr, Faulkner and Peaker, 1982; Faulkner and Pollock, 1990 a). Recently, it has been shown that the gut hormone, GLP-1, is insulinotrophic when administered to fasted sheep given intravenous glucose (Faulkner and Pollock, 1991).

Insulin has been implicated as a causal factor in certain obese states in ruminants (McCann, Bergman and Beerman, 1992); thus, indirectly, GIP may be an important determinant for adipose deposition. Furthermore, in simple-stomached animals GIP has direct effects on lipid metabolism which augment its insulinotrophic action. GIP increases both the rate of fatty acid synthesis (Oben *et al.*, 1989) and of fatty acid incorporation into adipose tissue (Beck and Max, 1987), enhances insulin receptor affinity in adipocytes (Starich *et al.*, 1985) and stimulates LPL activity in both cultured 3T3-L1 mouse preadipocytes (Eckel *et al.*, 1978) and rat adipose explants (Knapper *et al.*, 1993).

Antilipolytic properties have also been ascribed to GIP because of its inhibitory effect on glucagon-stimulated lipolysis (Dupre *et al.*, 1976; Ebert and Brown, 1976). However, since acetate does not appear to stimulate GIP release (Experiment 4.1), being absorbed from the rumen where K cells are not present, GIP may not be involved in the regulation of acetate utilization.

The aims of this study were (1) to investigate the insulin-releasing effect of GIP in fasted sheep, using a similar approach to that described by Faulkner and Pollock (1991), (2) to measure the incorporation of radiolabelled glucose into extractable fatty acids in rat adipose tissue explants incubated with different GIP preparations in order to identify the most potent preparation and (3) to use this GIP preparation to investigate the lipogenic effects of GIP in ovine adipose tissue.

## EXPERIMENTAL

### EXPERIMENT 5.1. EFFECT OF GIP ON INSULIN SECRETION IN STARVED SHEEP

#### Animals and their management

Six Finn-Dorset Horn wether sheep aged 9 months were used. In the period leading up to the experiment, the animals were fed 600g/day concentrates (goat mix 1, Edinburgh School of Agriculture: crude protein 165 g/kg, metabolizable energy 12.5 MJ/kg) and approximately 1600 g hay/day. Water was available *ad libitum*.

#### Experimental procedure

A polyethylene catheter (Internal diameter 0.8 mm; Dural plastics, Dural, Australia) was placed in the jugular vein the day before the start of the experiment. Injections were delivered and blood samples taken through this catheter. After a 24-hour fast, three sheep were given glucose only and three were given glucose plus 7.5 $\mu$g porcine GIP

(Peninsula, St Helens, U.K.). Three hours later (at 27 hours after feeding) treatments were reversed. GIP was aliquoted in sterile saline containing 10 % BSA and transferred to a 1 ml syringe, mixed with 1 ml of withdrawn blood, and injected via the jugular catheter. Glucose (5 g) was administered intravenously as a 50 % (w/v) solution in sterile water and flushed in with 3 ml saline.

Two blood samples (2.5 ml) were withdrawn prior to glucose administration. Further samples were taken at 1, 5, 10, 20, 30 and 45 minutes after glucose injection. Blood was mixed immediately with heparin (10 units/ml) and aprotinin (1000 KIU/ml), centrifuged at 8800 *g* for five minutes in a bench top centrifuge (Eppendorf, Hamburg, Germany) and the plasma stored at -20°C until analysed for GIP and glucose as described in Chapter 2. Blood taken for the measurement of insulin concentration was allowed to clot at room temperature for 2.5 hours, centrifuged, and serum removed and stored at -20°C until analysed as described in Chapter 2.

**Statistical analysis**

Data are given as means and S.E.D. Statistical analysis was by analysis of variance. Statistical significance was determined using variance ratios or Students t-tests as appropriate. The half-life of GIP was calculated for each animal from the gradient of the graph of log GIP concentration against time. Mean half-life and S.E.M. of values for six animals were calculated.

## EXPERIMENT 5.2.a. LIPOGENIC EFFECT OF GIP IN RAT ADIPOSE TISSUE

**Animals and their management**

Young male (100 g) Wistar rats (A.Tuck and Son, Rayleigh, Essex, U.K.) were allowed *ad iibitum* access to Labsure irradiated diet (Labsure, Poole, Dorset, U.K.) and water.

**Experimental procedure**

Explants from rat epididymal adipose tissue, prepared as described in Chapter 2, were incubated with 0.125 $\mu$Ci D-(U-$^{14}$C) glucose (Amersham International, Amersham, U.K.) for 2.5 hours at 37°C in Krebs-Bicarbonate buffer, pH 7.3, containing 5.5 mM glucose, and one of two preparations of synthetic porcine GIP (Peninsula, St Helens, U.K. or Sigma, Poole, U.K.) or natural porcine GIP (Gift from L.Morgan, University of Surrey, U.K.) at concentrations of 0, 15 and 25 ng/ml. Explants were also incubated with natural bovine insulin (Sigma, Poole, U.K.) at concentrations of 0, 1, 10 and 100 ng/ml. Rates of fatty acid synthesis were determined from the amount of radiolabelled glucose incorporated into saponifiable fatty acids (after total lipid extraction), as described in Chapter 2.

## EXPERIMENT 5.2.b. LIPOGENIC EFFECT OF GIP IN OVINE ADIPOSE TISSUE

**Animals and their management**

The sheep used in the experiment were nine month-old cross-bred Finn-Dorset Horn wether lambs given *ad libitum* access to hay plus 600 g/day of concentrates (goat mix 1, Edinburgh School of Agriculture: crude protein 165 g/kg, metabolizable energy 12.5 MJ/kg) in two equal meals at 08.00 and 16.00 hours.

**Experimental procedure**

Explants prepared, as described in Chapter 2, from ovine subcutaneous adipose tissue were incubated with 0.125 $\mu$Ci sodium (1-$^{14}$C)-acetate (Amersham International, Amersham, U.K.) for 24 hours at 37°C in Medium 199 supplemented with synthetic porcine GIP (Peninsula, St Helens, U.K.) at concentrations of 0, 6, 12, 25, 50, and 100 ng/ml or natural bovine insulin (Sigma, Poole, U.K.) at concentrations of 0, 1, 10 and 100 ng/ml. Rates of fatty acid synthesis

were determined from the amount of incorporation of radiolabelled acetate into fatty acids (after total lipid extraction), as described in Chapter 2.

**Statistical analysis**

Data are given as means with S.E.D. Statistical analysis was by one-way analysis of variance and significance was determined by variance ratios and t-tests.

## RESULTS

### EXPERIMENT 5.1. EFFECT OF GIP ON INSULIN SECRETION IN STARVED SHEEP

As shown in Figure 5.1.a, in sheep injected with glucose alone, GIP concentrations were comparable ($P > 0.05$) to the basal level for the duration of the experiment. After GIP injection, circulating levels of GIP increased significantly ($P < 0.001$) from the basal concentration reaching 4 ng/ml after GIP injection, then declined from 5 to 45 minutes, but remained significantly higher ($P < 0.001$) than basal concentrations throughout the sampling period. The biological half-life of GIP was estimated to be 10.9 minutes (S.E.M. = 1.38).

Plasma glucose concentrations increased ($P < 0.01$) above the basal level to approximately 8 mM after glucose injection, irrespective of whether or not GIP was also administered (Figure 5.1.b). The peak glucose concentrations and subsequent rates of decline when glucose was given alone were not significantly different from those when glucose was given with GIP. Glucose concentrations were still elevated above basal levels at the end of the sampling period ($P < 0.05$).

Plasma insulin concentrations increased ($P < 0.001$) within 10 minutes of the intravenous glucose injection in all sheep (Figure 5.1.c). Insulin levels remained significantly above the pre-injection

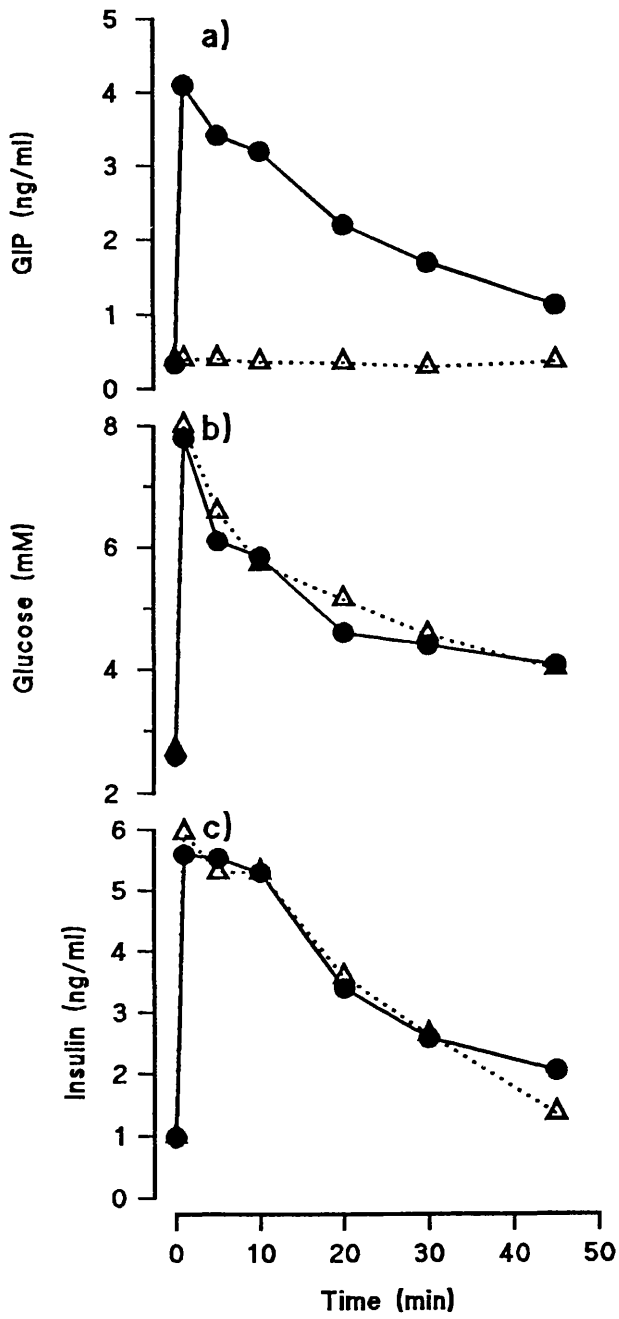



**Figure 5.1.** Changes in concentrations of (a) plasma GIP, (b) plasma glucose and (c) serum insulin in fasted sheep after intravenous injection of glucose (5 g) with (●—●) or without (△···△) GIP (7.5 $\mu$g) at time 0. Data are means of 6 animals, with S.E.D., within and between levels of GIP injections, of (a) 0.18 and 0.20 ng/ml (b) 0.58 and 0.59 mM, and (c) 0.52 and 0.62 ng/ml.

concentration for the duration of the experiment, except at 45 minutes after injection in sheep given glucose alone. Throughout the sampling period plasma insulin concentrations in the sheep when glucose was given with GIP were not significantly different from those when glucose was administered alone.

EXPERIMENT 5.2.a. LIPOGENIC EFFECT OF GIP IN RAT ADIPOSE TISSUE

Incubation of rat adipose explants for 2.5 hours at 37°C with insulin concentrations ranging from 1 to 100 ng/ml resulted in a dose-dependent stimulation of lipogenesis with a maximum increase 7-fold above basal (Figure 5.2.a). Physiological levels of insulin at 1 ng/ml increased the lipogenic rate, with maximum stimulation achieved at 10 ng/ml. As shown in Figure 5.2.b., all three GIP preparations enhanced the incorporation rate of $^{14}$C-glucose into fatty acids at the two levels of hormone tested (15 and 25 ng/ml). The two synthetic porcine GIP preparations gave comparable effects, with both demonstrating a dose-related increase in the rate of lipogenesis. In contrast, both doses of natural porcine GIP induced comparable rates of lipogenesis, with the lower GIP level of 15 ng/ml giving greater stimulation than the same concentration for each synthetic peptide.

EXPERIMENT 5.2.b. LIPOGENIC EFFECT OF GIP IN OVINE ADIPOSE TISSUE

Incubation of ovine adipose tissue explants for 24 hours at 37°C with insulin concentrations ranging from 1 to 100 ng/ml enhanced lipogenesis in a dose-dependent manner (Figure 5.3). Physiological concentrations of insulin (1 ng/ml) significantly enhanced ($P < 0.05$) the rate of fatty acid synthesis, with no additional stimulation beyond the maximum response at a concentration of 10 ng/ml. Synthetic porcine GIP (Peninsula, St Helens, U.K.) gave only a weak stimulation of

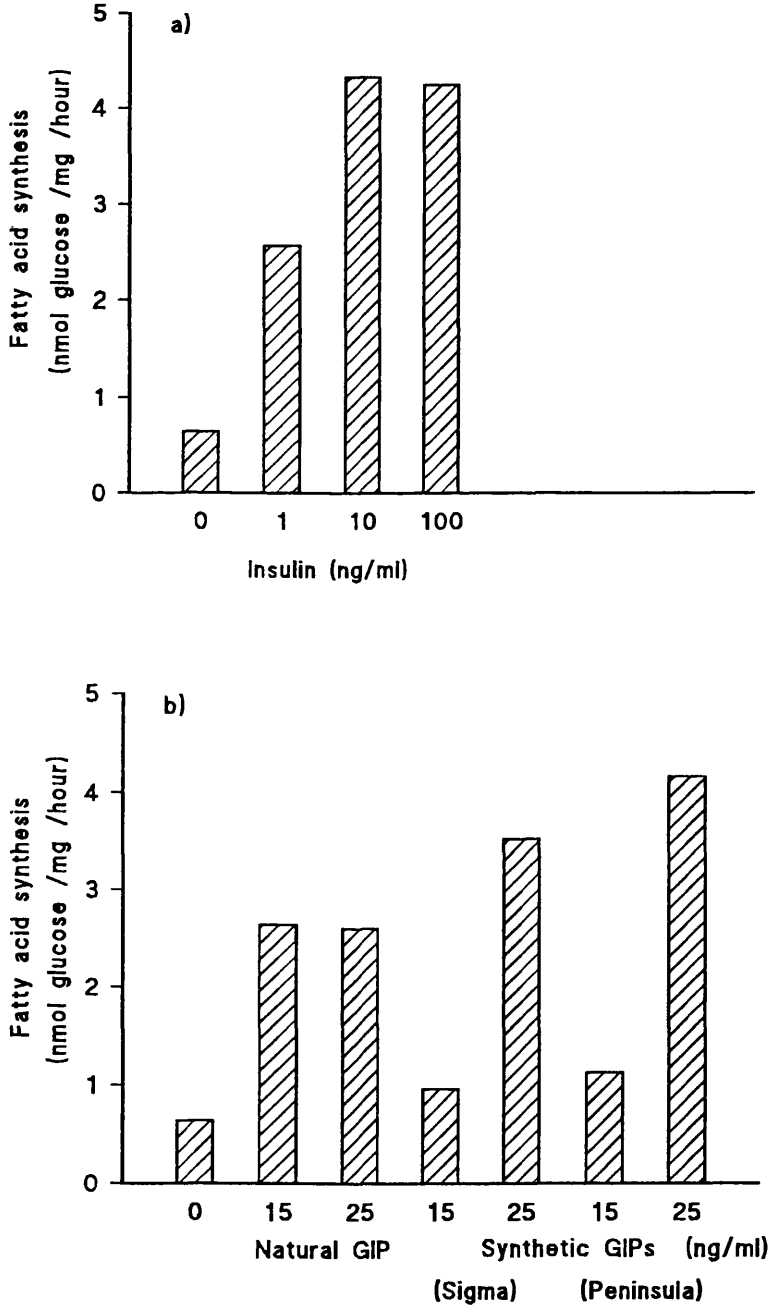



**Figure 5.2.** Stimulation of fatty acid synthesis (nmol glucose incorporated/mg wet weight/hour) by (a) insulin or b) GIP in rat epididymal adipose tissue explants (n=1).

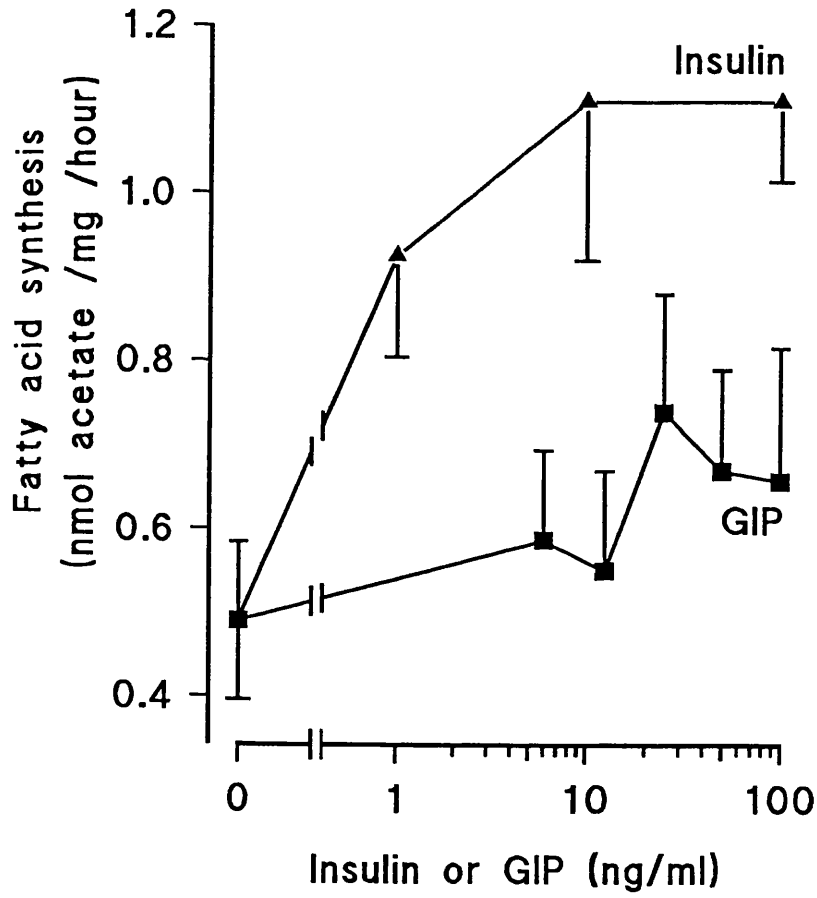



**Figure 5.3.** Stimulation of fatty acid synthesis (nmol acetate incorporated/mg wet weight/hour) by insulin (▲—▲) or GIP (■—■) in ovine subcutaneous adipose slices. Data are means, with S.E.M., of 5 animals.

lipogenesis. The rate of fatty acid synthesis tended to increase dose-dependently, although statistical significance was only achieved at 50 ng/ml ($P < 0.05$).

## DISCUSSION

The results of Experiment 5.1 demonstrated that exogenous GIP was ineffective in eliciting insulin secretion in sheep, even though the insulinotrophic effect of GIP is well documented in simple-stomached animals (Morgan, 1992). The possibility that porcine GIP was not biologically active in sheep is unlikely because the same source of GIP was shown to stimulate lipogenesis in ovine adipose tissue in Experiment 5.2.b. Moreover, the increase in plasma GIP concentrations and the degree of glycaemia attained after intravenously-administered GIP (7.5$\mu$g) and glucose (5 g) was within a range known to be effective in stimulating insulin release from pancreatic islets and perfused pancreas in rats (Siegel and Creutzfeldt, 1985; Pederson and Brown, 1976) and *in vivo* in man (Dupre *et al.*, 1973). Peak changes in GIP concentration exceeded those seen during the postprandial period in sheep or goats (Chapter 3 and 4) or after oral glucose administration in man (Cataland *et al.*, 1974; Anderson *et al.*, 1978) and ob/ob mice (Flatt, Kwasowski, Bailey and Bailey, 1989) but for much of the duration of the experiment were in the range seen after feeding.

The failure to detect an insulinotrophic effect of GIP in sheep (Experiment 5.1) may be related to the greater than anticipated degree of glycaemia obtained with the intravenous glucose load (5 g), which exceeded those reported by Faulkner and Pollock (1991) in a comparable experiment in which GLP-1 was shown to be insulinotrophic in sheep. Further studies using a smaller intravenous glucose load to give a more appropriate level of glycaemia and hence a moderate increase in insulin secretion would be a more appropriate test of the ability of GIP to augment glucose-stimulated insulin secretion. As such, the data reported

here, which fail to demonstrate any augmentation of glucose-stimulated insulin secretion, should be treated with caution. However, in view of the fact that absorption of glucose from the small intestine consistently fails to elicit GIP release in sheep or goats (Chapters 3 and 4) whilst it is effective in other species (Anderson *et al.*, 1978; Flatt *et al.*, 1989) a role for GIP as an incretin seems less likely in ruminant species. It remains possible that the insulin-releasing action of GIP in ruminants could be dependent upon synergism with other gut hormones (Zawalich, 1988). Reduced circulating concentrations of these hormones in the fasted state could account for the failure to detect an insulinotrophic effect of GIP in ruminants.

The biological half-life of exogenous GIP infused in sheep was estimated to be 10.9 minutes (S.E.M. = 1.38), which contrasts with the reported values of approximately 20 minutes (Brown, Dryburgh, Ross and Dupre, 1975; Sarson *et al.*, 1982) and of greater than 30 minutes in humans (Kreymann *et al.*, 1987).

The results of Experiment 5.2.a. confirmed that all three GIP preparations tested were biologically active, as shown by their lipogenic action in rat adipose tissue. An insulin-like effect of GIP was also demonstrated in ovine adipose tissue in Experiment 5.3 although when compared with insulin, GIP does not appear to be a major factor in stimulating the incorporation of acetate into fatty acids. However, a similar study by Haji Baba and Buttery (1991) reported a strong lipogenic effect of GIP in ovine adipose tissue. Also, direct insulin-like effects of GIP have been demonstrated recently in ovine adipose tissue perfusates *in vivo* using microdialysis; intravenous GIP infusion decreased the concentration of glucose in the perfusate, consistent with an increase in lipogenesis (Martin, Faulkner and Thompson, 1993). A role for GIP in lipid metabolism in ruminants is consistent with the observed GIP secretion in response to fat absorption in the young ruminant (Chapter 3) and the timing of its release postprandially in adult sheep (Chapter 4).

Although the lipogenic effect of GIP appears to be weak compared with that of insulin in the ruminant, GIP may play an important role in the clearance of long-chain fatty acids during the post prandial period. However, the involvement of GIP in these aspects of lipid metabolism has yet to be investigated in ruminant species.

# CHAPTER 6

## General discussion

The aim of the studies presented in this thesis was to investigate the role of GIP in ruminant physiology. Specifically, the objectives were to determine whether circulating concentrations of GIP are responsive to nutrient ingestion, to identify specific GIP secretagogues, and to examine nutrient-induced GIP secretion in both the development of obesity and in lactation, and finally to examine possible effects of GIP on insulin secretion and adipose tissue metabolism.

In simple-stomached animals, GIP is secreted from K cells in the small intestine in response to an oral glucose load (Cataland *et al.*, 1974). The response is dependent upon active transport of the monosaccharide across the brushborder membrane (Sykes *et al.*, 1980). Thus, phlorizin, a competitive inhibitor of the $Na^+$/glucose transporter, curtails glucose-stimulated GIP release (Creutzfeldt and Ebert, 1977). Furthermore, studies with glucose analogues have shown the GIP response to actively-absorbed sugars to be independent of their metabolism or passage across the basolateral membrane of the enterocyte (Sykes *et al.*, 1980; Flatt *et al.*, 1989).

The differences in digestion between simple-stomached and ruminant animals, notably the small amount of glucose absorbed from the small intestine in ruminants (Merchen, 1988), raise the possibility that there are differences in the regulation of GIP secretion between these species.

In marked contrast to the situation in simple-stomached animals, in none of the studies reported in this thesis did glucose have any effect on GIP release in ruminants. Just how this effect is lost is unclear but presumably the K cell has lost its sensitivity to glucose, possibly through loss of the glucose transporter on the brushborder membrane. Thus, in ruminant animals, the absence of a glucose transporter on the K cell brushborder membrane with maintenance of glucose transport in enterocytes could be consistent with the ability of ruminants to absorb glucose, but to fail to respond in terms of GIP secretion. It seems unlikely that loss of the glucose transporter from the K cell occurs

during maturation of the animal because glucose absorption had no effect on GIP secretion in the pre-ruminant goat. This is consistent with the absence of a GIP response to glucose being attributable to evolutionary rather than environmental factors.

Whilst glucose is the main regulator of insulin release in simple-stomached animals, several gut hormones have been implicated as incretins because of their ability to augment glucose-induced insulin release in pancreatic $\beta$-cells (Morgan, 1992). GIP augments glucose-induced insulin secretion and is considered a major component of the entero-insular axis. GIP has an insulinotrophic effect in perifused islets of Langerhans (Siegel and Creutzfeldt, 1985; Zawalich, 1988), in the isolated perfused pancreas (Clark *et al.*, 1989) and *in vivo* in both animals (Ahren and Lundquist, 1983) and humans (Nauck *et al.*, 1993). The insulinotrophic action of GIP occurs in a dose-related manner (Pederson and Brown, 1976) and is dependent on a glucose threshold of approximately 1 - 2 mM above basal (Elahi *et al.*, 1979; Nauck *et al.*, 1991), below which GIP fails to stimulate insulin secretion.

The exact mechanism of action for most incretins remains to be established, but it has been shown that GIP binds to specific receptors on the pancreatic $\beta$-cell (Maletti, Portha, Carlquist, Kergoat, Laburthe, Marie and Rosselin, 1984) activating adenylate cyclase and potentiating glucose-stimulated insulin release by gating voltage-dependent channels in the membrane to increase intracellular $Ca^{2+}$ (Lu, Wheeler, Leng and Boyd, 1993). GLP-1 appears to act in the same way, whereas CCK binds to its specific receptor, activating phospholipase C leading to amplification of the $Ca^{2+}$ signal for insulin release (Berggren, Rorsman, Efendic, Ostenson, Flatt, Nilsson, Arkhammar and Juntti-Berggren, 1992). Therefore, synergistic effects observed with incretin hormones can be explained by interaction of these different mechanisms of action.

The results presented in this thesis indicate that GIP is not insulinotrophic in ruminants. It seems likely that the GIP preparation used was biologically active because the same source was shown to

stimulate lipogenesis in ovine adipose tissue. Furthermore, a comparable study has shown the gut hormone GLP-1 to be insulinotrophic in sheep whereas GIP was not, although the author acknowledged that the biological activity of GIP used in the study was not assessed (Faulkner, 1990). The failure to demonstrate an augmented insulin-releasing effect with intravenous GIP and glucose injection in sheep in the present study could also be related to the level of glycaemia, which exceeded that reported by Faulkner and Pollock (1991). Further studies with a reduced intravenous glucose load and a range of GIP concentrations achieved by continuous intravenous infusion would be a more appropriate test of the ability of GIP to augment glucose-stimulated insulin secretion. Alternatively, an *in vitro* approach using isolated ovine islets of Langerhans could enable a more extensive investigation of the potential insulinotrophic effect of GIP in sheep. Although GIP may not have a direct insulinotrophic role it could serve to prime the pancreas for the insulinotrophic effect of other gastrointestinal hormones. Examination of this hypothesis could be achieved by intravenously infusing GIP alone or with other potential incretins such as GLP-1, and monitoring the subsequent insulin response to an intravenous glucose load.

A lack of an insulinotrophic effect of GIP in the ruminant could be attributed to the absence of a GIP receptor on the pancreatic $\beta$-cell. Alternatively, because the ruminant absorbs only small amounts of glucose from the small intestine (Merchen, 1988), and propionate is the principal glucogenic nutrient absorbed by ruminants (Thomas and Rook, 1983), a more appropriate test for the ability of GIP to augment insulin secretion could be to infuse GIP intravenously with propionate.

In simple-stomached animals, GIP is also secreted in response to absorption of fat from the small intestine (Falko *et al.*, 1975). Specific fatty acids differ in their ability to elicit GIP release, for example GIP secretion is stimulated by long-chain fatty acids but not medium- or short-chain fatty acids (Ross and Shaffer, 1981; Kwasowski *et al.*, 1985). Esterification of long-chain fatty acids in the enterocyte appears

to be a pre-requisite for GIP secretion. Thus, Pluronic L-81, a hydrophobic surfactant which blocks chylomicron formation, inhibits GIP release during long-chain fatty acid absorption (Tso *et al.*, 1981; Ebert and Creutzfeldt, 1984).

In line with observations in simple-stomached animals, the results presented in this thesis demonstrate that fat is a potent GIP secretagogue in ruminants. Future studies could be directed at examining the effect of fatty acid chain length and degree of saturation on GIP secretion.

Fat-induced GIP secretion in goat kids and its release postprandially in adult sheep is consistent with a role for GIP in the regulation of ruminant lipid metabolism. GIP has direct effects on several aspects of lipid metabolism in simple-stomached animals. For example, GIP has been shown to increase the rates of both fatty acid synthesis (Oben *et al.*, 1989) and fatty acid incorporation into rat adipose tissue (Beck and Max, 1987), and to stimulate LPL activity in cultured mouse pre-adipocytes (Eckel *et al.*, 1978) and rat adipose tissue explants (Knapper *et al.*, 1993). Furthermore, GIP enhances insulin receptor affinity in adipocytes (Starich *et al.*, 1985), promotes the clearance of chylomicron triacylglycerol (Wasada *et al.*, 1981) and has an inhibitory effect on glucagon-stimulated lipolysis (Dupre *et al.*, 1976) by selectively blocking glucagon activation of adenylate cyclase (Ebert and Brown, 1976).

An insulin-like effect of GIP was shown in ovine adipose tissue in studies reported here, although when compared with insulin, GIP did not appear to be a major factor in regulating fatty acid synthesis in the ruminant. In contrast, Haji Baba and Buttery (1991) reported a strong lipogenic effect of GIP in ovine adipose tissue. This apparent contradiction could be attributed to their use of perirenal rather than subcutaneous adipose tissue which was used in the studies presented in this thesis. Alternatively, the relative lipogenic effect of GIP compared with that of insulin could have been overestimated because of poor sensitivity of the perirenal adipose tissue to insulin.

More recently, direct insulin-like effects of GIP have been shown in ovine adipose tissue perfusates *in vivo* using microdialysis, a technique that eliminates many of the disadvantages associated with explants because the tissue remains in its natural environment. The effect of intravenous GIP infusion was to induce a decrease in the concentration of glucose and glycerol in the perfusate (Martin, Faulkner and Thompson, 1993). This was consistent with studies in simple-stomached animals in which GIP enhanced lipogenesis (Oben *et al.*,1989) and inhibited lipolysis (Dupre *et al.*, 1976).

GIP may play an important role in the clearance of long-chain fatty acids during the post-prandial period. For example, GIP has been shown to enhance disposal of chylomicrons from the circulation in dogs (Wasada *et al.*, 1981), and to stimulate LPL activity in both cultured mouse pre-adipocytes (Eckel *et al.*, 1978) and rat adipose explants (Knapper *et al.*, 1993). Also, GIP and insulin may be synergistic in their stimulation of LPL activity in rat adipose explants (Knapper *et al.*, 1993). Furthermore, LPL activity can be modified by dietary intake in man (Romsos and Leveille, 1975), indicating the possible involvement of gut hormones in the regulation of LPL. Results presented in this thesis demonstrate that GIP levels are increased in obese sheep, and this may have encouraged triacylglycerol uptake into adipose tissue. In view of this, preliminary studies were conducted in ovine adipose explants with the aim of evaluating the possible effect of GIP on LPL activity but there was unfortunately insufficient time to complete these studies.

In sheep, the metabolic status of the animal is known to influence adipose tissue metabolism. Lactation is associated with an increase in lipid mobilization and reduced lipogenesis in adipose tissue, resulting in an increased supply of fatty acids to the mammary gland to support milk production (Vernon and Flint, 1983). Lactation leads to large increases in food intake, similar to those that occur in obesity, and it might be expected that GIP secretion would be increased in such circumstances. Insulin secretion however, which is also normally responsive to food

intake, is decreased during lactation; this is thought to play an important part in nutrient partitioning by suppressing normal anabolic processes and making nutrients available for milk production. In fact, GIP concentrations were increased in lactating sheep which was presumably related to the higher level of dietary intake.

As the sensitivity of tissues to hormonal stimulation is modified during lactation (Vernon and Taylor, 1988), it is possible that GIP secretion evoked in lactating animals regulates lipid metabolism not in adipose tissue but in the mammary gland. This would also favour nutrient partitioning towards milk production. Studies using mammary gland explants could determine whether GIP has a stimulatory effect on mammary LPL activity or if GIP receptors exist on mammary secretory cells. If GIP receptors do occur on mammary cells it would be interesting to assess whether reciprocal changes in GIP receptors occur on mammary and adipose cells in a fashion analogous to changes in insulin receptors (Flint, 1982). Such changes may play an additional role in regulating tissue sensitivity to favour nutrient uptake in mammary gland whilst restricting it in adipose tissue. There is evidence that circulating GIP concentration modulates tissue sensitivity; studies in rats have shown that elevated basal GIP concentrations can reduce sensitivity of the $\beta$-cell to GIP and that changes in sensitivity are apparently mediated by alterations at the receptor (Pederson, Innis, Buchan, Chan and Brown, 1985). Furthermore, in humans with type-2 diabetes, and high basal GIP concentrations, the sensitivity of the $\beta$-cell to GIP is reduced (Nauck, Stockmann, Ebert and Creutzfeldt, 1986).

In summary, circulating concentrations of GIP in the ruminant were responsive to nutrient ingestion. Glucose absorption had no effect on GIP release, whereas fat was a potent GIP secretagogue. In the different metabolic states of obesity and lactation, hyperphagia was associated with increased GIP secretion. In ovine adipose tissue, GIP was shown to have an insulin-like effect. In contrast to its effect in simple-stomached animals, GIP was not insulinotrophic in the ruminant.

These findings were consistent with a role for GIP in ruminant lipid metabolism.

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