The role of the vagal innervation of the stomach (abomasum and pylorus) and intestine (duodenum) in insulin and oxytocin release in sheep

A thesis presented in partial fulfilment of the
requirements for the degree of Doctor of Philosophy in
Animal Science at Massey University, Palmerston North,
New Zealand

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1998

ABSTRACT

Although mechanisms regulating nutrient partitioning and milk synthesis are not fully understood in ruminants, recent studies in lactating monogastric animals have shown that the vagus nerve modulates secretion of various hormones that are implicated in the short- and long-term control of nutrient partitioning. Therefore, the overall aim of this study was to examine the role of the vagal innervation of the GI tract on insulin and oxytocin release and milk yield in sheep. In a series of experiments described in this thesis, the effect of vagotomy was studied in ewes and wethers by comparing the responses of vagotomized animals (i.e. abomasal, pyloric, duodenal and hepatic branches sectioned) with control (sham-operated) animals.

Insulin release in response to a bolus injection of glucose was studied in lactating ewes (Chapters 2 and 4) or wethers (Chapters 3 and 5). The differences in responses were not significant in the experiments described in Chapters 3 and 4. However, the release of insulin from the pancreas in response to glucose injection was significantly ($P < 0.05$) suppressed in the vagotomized animals used in the experiments described in Chapters 2 and 5. Moreover in the experiments with wethers (Chapter 5), insulin secretion in response to glucose bolus injection was significantly ($P < 0.05$) higher when administered 2 h (i.e. fed state) following feeding than 22 h (i.e. fasting state). In addition, postprandial insulin concentrations were significantly ($P < 0.05$) lower in the vagotomized wethers than in the sham-operated wethers, but insulin secretion in the vagotomized wethers was apparently unaffected by plane of nutrition, despite significantly ($P < 0.05$) higher blood glucose levels in wethers on the HP intake. The insulin concentrations were, however, higher ($P < 0.05$) in the control group of wethers fed on the high plane (HP) of nutrition than those fed on the low plane (LP) of nutrition (Chapter 5).

Insulin was released in response to the sight and/or ingestion of food, cephalic phase insulin release (CPIR), without any significant changes in blood glucose concentrations. However, the increase in insulin concentration was significantly ($P <$

0.05) suppressed in both vagotomized wethers and ewes in comparison with control animals (Chapters 5 and 6).

Suckling increased plasma insulin concentrations in the sham-operated ewes but not in the vagotomized ewes (Chapter 6), although the difference in the concentrations between the two groups was not statistically significant ($P < 0.09$).

Milk and fat yields were significantly ($P < 0.05$) reduced for one day and two days, respectively, in the vagotomized ewes compared with those of sham-operated controls, but was restored over the next 2-3 days (Chapter 2). Milk yield was not different between the two treatment groups in the second study (Chapter 4).

Suckling-associated plasma oxytocin concentrations were significantly ($P < 0.01$) lower in the vagotomized ewes than in the sham-operated control ewes, although the difference was not statistically significant when corrected for baseline values (Chapter 4). In the next experiment (Chapter 6), oxytocin concentrations between the two treatment groups of ewes were not significantly different. However, in this experiment, suckling caused a significant ($P < 0.05$) increase in oxytocin concentrations from the baseline values in the sham-operated ewes fed and suckled simultaneously but not in the vagotomized ewes fed and suckled simultaneously.

Vagotomy significantly ($P < 0.05$) increased digestibility of dry matter and nitrogen in wethers, although food intake was not different between the two treatment groups (Chapter 3).

In conclusion, the findings in wethers (Chapter 5) agree with those in lactating ewes (Chapters 2 and 4) and, indicated that the effect of vagotomy on insulin release in response to glucose injection is more apparent over a short period (i.e. 2-4 h; Chapters 2 and 5) following feeding than after a longer period (i.e. 6-22 h; Chapters 4 and 5). This suggested that the pancreatic β-cells are more sensitive soon after feeding, because the vagal inputs reaching the β-cells from the GI tract are higher due to the recent consumption of food. The finding that post-prandial insulin concentrations in the

vagotomized animals of HP group were significantly reduced, despite their significantly higher blood glucose levels, provide further evidence that the vagus nerve is a major determinant for sensitizing pancreatic β-cells. Furthermore the vagal innervation of the GI tract plays a major role in CPIR, and also appears to play an important role in insulin release during suckling in sheep. The concentrations of oxytocin measured in these experiments suggest that vagotomy interferes with oxytocin secretion although differences between vagotomized and sham operated ewes were often non significant. However, the data suggest that feeding stimulates OT secretion. It is possible that the failure to achieve consistent differences in milk ejection and hence removal in these studies may have been partly masked because of the anatomical features of the mammary gland of the ewe. Finally the activity of the vagus nerve influences the digestibility of dry matter and nitrogen in sheep.

ACKNOWLEDGEMENTS

My sincere gratitude is expressed to my supervisors, Drs Duncan Mackenzie, Tricia Harris, Gordon Reynolds and Steve Davis for their commitment, critical guidance, wise counsel and encouragement throughout the study.

I would also like to thank Assistant Vice Chancellor Professor Stuart N McCutcheon and Pro Vice Chancellor Professor Robert D Anderson for giving me an opportunity to undertake this study in the then Department of Animal Science, Massey University, and specially Professor McCutcheon for his support during my transition from the Masters study to PhD study.

The support and encouragement of the postgraduate students and staff in the Department of Animal Science at Massey University and also my friends Ken, Nalaka, Penny, Reddy, Priya, Shankar, Jeya and Arturo for helping my experimental work, is gratefully acknowledged.

My sincere gratitude is also extended to Dr Phillip Pearce, Ms Yvette Cottam, Ms Margaret Scott, Mr Brett Guthrie, Mr Barry Parlane, Mr Guy Hessell and Mr Bruce Sinclair for their excellent technical assistance, and Dr Patrick Morel for his advice on matters statistical. I would also express my thanks to Ms Kathy Morton for helping in the radioimmunoassays. The commitment of Tricia and Bruce for supplying anything and everything at anytime for my experiments is gratefully acknowledged.

Special thanks are also due to Ms Margaret Scott and Dr Vernon Choy for their valuable advice to develop and setting up an oxytocin radioimmunoassay, and Dr Vernon Choy also for supplying oxytocin antibody.

I would also express my sincere thanks to the Scholarships Committee, for awarding me a Massey University PhD Scholarship (1995 to 1998) and G. O. Anstiss Scholarship (1998).

The support of the staff of the Massey University Library, and the Foundation of Research, Science and Technology for providing me research funds is also gratefully acknowledged.

Special thanks are due to my mother and brothers for their love, support and encouragement. A very special thank is due to my late father for always believing in me and teaching me that I can do anything by believing in myself. Equal gratitude is extended to my father-in-law and mother-in-law for their love, unfailing support and encouragement.

Finally, I am indebted to my wife, Ayoni, and my sons, Ishan and Vimukthi, for their unconditional love and endless patience without which I could not have achieved so far.

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LIST OF ABBREVIATIONS

ADV	Abomasal and duodenal vagotomy
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BL	Balance
bw	Body weight
CB 154	2-Br-Ergocriptine
CCK	Cholecystokinin
CNS	Central nervous system
CPIR	Cephalic phase insulin release
CT-HRP	Cholera toxin horseradish peroxidase
DM	Dry matter
DMH	Dorsomedial hypothalamus
DMN	Dorsal motor nucleus
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
h	Hour(s)
HADV	Hepatic, abomasal and duodenal vagotomy
HP	High plane of nutrition
ID	Internal diameter
i.c.v.	Intracerebrovascular
i.m.	Intramuscular
i.p.	Intraportal
i.v.	Intravenous
K	The glucose clearance constant
kg	Kilogram
LP	Low plane of nutrition

ME	Metabolizable energy
min	Minutes
MJ	Megajoules
mmol	Millimole
MSG	Monosodium glutamate
N	Nitrogen
NEFA	Non-esterified fatty acids
ng	Nanogram
NTS	Nucleus tractus solitarius
OD	Outer diameter
OT	Oxytocin
PF	Post-feeding
pg	Picogram
PO	Post-operation
PRL	Prolactin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
s.c.	Subcutaneous
sec	Seconds
SO	Sham-operation
SON	Supra optic nucleus
SS	Somatostatin
VFA	Volatile fatty acids
VIP	Vasoactive intestinal polypeptide
VMH	Ventromedial Hypothalamus

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CHAPTER 1: INTRODUCTION

1.1 PREAMBLE

Lactation is the phase of reproduction during which mammals produce milk in the mammary gland for nourishing their young. The functional mammary gland is one of the most highly differentiated and metabolically active tissues in the body of lactating animals. Lactation requires a high proportion of the nutrients available in the body to be diverted from the peripheral tissues towards the mammary gland for milk synthesis. Those ruminant species that have been domesticated and bred for dairy production have a high potential for milk production. Therefore, during lactation, a well-coordinated system must be present to meet the demand for nutrients for milk synthesis by the mammary gland and also to maintain the vital functions of the body tissues. These vital functions are changing continuously on a short-term basis (homeostasis) while different physiological states like lactation may require changes over a long-term (homeorhesis) (Bauman and Currie, 1980).

Homeostasis is a concept of multiple compensatory mechanisms that maintain the physiological equilibrium of body functions in response to changes in external environment. For example, movements of metabolites, such as carbohydrates, protein and fat, between tissues are happening continuously. A change in external environment, for example consumption of a meal, leads to an alteration in the relative influence of various homeostatic signals which, in turn, increases the relative magnitude of the anabolic process of the cycle. On the other hand, when the nutrient intake is diminished, the relative magnitude of the catabolic process of the cycle is increased. Thus, homeostatic control is operating throughout, irrespective of the physiological status of the animal, to maintain vital body functions and to maintain equilibrium in conditions such as stress or changes in circulating metabolites associated with food intake.

During lactation, a large demand for nutrients created by the active mammary gland is met primarily by nutrients absorbed from the gastrointestinal (GI) tract or released from body reserves (e.g. adipose tissue). Thus, an increased rate of delivery of these nutrients towards the mammary gland is achieved by alterations in whole body

metabolism. This is the basis of the concept of nutrient homeorhesis in which a higher proportion of nutrients is preferentially partitioned from peripheral tissues to the mammary gland to support the requirements for milk synthesis (Bauman and Currie, 1980).

Insulin, a major anabolic hormone, plays a key role in hormonal control of homeostasis in ruminants (Brockman, 1978). Although insulin may not have any direct effect on the ruminant mammary gland, it regulates the supply of substrates to the mammary gland by altering peripheral extramammary metabolism (Metcalf *et al.*, 1991; Tesseraud *et al.*, 1992). Glucagon, a major catabolic hormone, is also involved in minute-to-minute control of nutrient metabolism in ruminants but it seems to have an insignificant influence on basal glucose production, protein and lipid metabolism in lactating ruminants (Brockman and Laarveld, 1986). Glucocorticoids cause a decrease in peripheral uptake of glucose and increase in gluconeogenesis (Reilly and Black, 1973; Reilly and Ford, 1974) while catecholamines provide immediate supplies of glucose and lipid fuels in times of stress and hypoglycaemia (Brockman, 1985), by increasing lipolysis and glycogenolysis (Blum *et al.*, 1982; Thomson *et al.*, 1978).

Growth hormone (GH) has been shown to be essential for the maintenance of lactation and it has a major role in the homeorhetic control of nutrient partitioning (Bauman and Currie, 1980). Studies in the 1950's and 1970's, involving acute or chronic bovine GH treatment, showed that injection of exogenous GH increased milk production (Hutton, 1957; Hart *et al.*, 1979). Many later studies showed that administration of exogenous GH over a short period, increased the yield of milk and its components in dairy cows (Peel and Bauman, 1987; Peel *et al.*, 1983, 1982, 1981), sheep (McDowell *et al.*, 1988; Hart *et al.*, 1985) and goats (Mepham *et al.*, 1984; Hart *et al.*, 1980). Studies in which GH was administered over a long period of time also increased milk yield in dairy cows (Bauman *et al.*, 1985; Peel *et al.*, 1985) and sheep (Sandles *et al.*, 1988). Growth hormone stimulates lipolysis by increasing the sensitivity of adipose tissues to catecholamines and thereby ensuring an adequate supply of non-esterified fatty acids (NEFA) to the mammary gland for milk fat synthesis (Bauman and McCutcheon, 1986). Thus, elevated GH concentrations during lactation may have a role in facilitating the transfer of energy in the form of fatty acids from peripheral tissues to the lactating

mammary gland. Further, increases in feed intake during GH treatment are a long term metabolic adaptation by which cows sustain milk yield.

The effect of prolactin (PRL) during established lactation in ruminants appears to differ considerably from that in non-ruminants. Lactation may be totally inhibited in non-ruminants by blocking the release of PRL, but the course of lactation is unaffected by such a blockade in cows (Akers *et al.*, 1981) or sheep (Hooley *et al.*, 1978), suggesting that PRL is not a part of homeorhetic control during established lactation in ruminants.

Although metabolic control of insulin secretion has been extensively studied (De Jong, 1982; Sano *et al.*, 1993; Sutton *et al.*, 1988; Horino *et al.*, 1968; McAtee and Trenkle, 1971), the mechanisms by which endogenous and exogenous nutrients stimulate insulin release in ruminants are still not well understood. Administration of volatile fatty acids (VFA) into the jugular or femoral vein caused an increased secretion of insulin in goats (De Jong, 1982), sheep (Sano *et al.*, 1993, 1995) and lambs (Bloom and Edwards, 1985). Administration of glucose into the jugular or saphenous vein caused an increased secretion of insulin in sheep (De Jong, 1982) and calves (Bloom and Edwards, 1981). Increases in insulin concentration in response to physiological doses of propionate given i.v. (Sano *et al.*, 1995) or into the rumen (Istasse *et al.*, 1987) were not preceded by any change in plasma glucose concentrations. This indicates that the release of insulin is unlikely to be mediated through a direct action of glucose produced from propionate in the liver. It is also unlikely, however, that propionate acts directly on the pancreatic β-cells to stimulate insulin secretion since the majority of the propionate absorbed through the GI tract and portal vein is removed by the liver (Bergman, 1975). Pre-treatment with atropine, a muscarinic blocker, abolished insulin secretion induced by i.v. infusion of propionate and butyrate in sheep (Sano *et al.*, 1993), butyrate in lambs (Bloom and Edwards, 1985) and glucose in calves (Bloom and Edwards, 1981). Furthermore pre-treatment with atropine inhibited insulin release in response to electrical stimulation of the vagus nerves in calves (Adrian *et al.*, 1983; Bloom and Edwards, 1981). Electrical stimulation of the vagus without atropine causes insulin release in sheep, pigs and baboons (Pierzynowski *et al.*, 1986; Holst *et al.*, 1981; Daniel and Henderson, 1967), and abdominal vagotomy prevents the rise of insulin in response to cervical vagal stimulation in rats (Berthoud and Powley, 1990; Berthoud *et al.*, 1990). These observations strongly

suggest that insulin secretion is mediated, in part at least, through the vagus nerve in monogastric animals and probably in ruminants as well.

An increased availability of nutrients to the ruminant mammary gland during lactation is not always associated with any increase in milk or milk solids yield. For example, systemic infusion of metabolites such as glucose and amino acids, combined with or without insulin infusion, failed to alter mammary metabolism in lactating goats (Tesseraud *et al.*, 1992; Hove, 1978a; Linzell, 1967), cows (Metcalf *et al.*, 1991; Laarveld *et al.*, 1981) and women (Neville *et al.*, 1993), indicating that the availability of substrates to the mammary gland may not be rate limiting for mammary metabolism and hence milk synthesis. In contrast, Griinari *et al.* (1997a) reported that although insulin infusion did not alter milk yield in cows, it did increase yield when combined with abomasal protein infusion. Based on these results, a question that needs to be answered is why milk yield is not affected when nutrients are infused directly into the systemic circulation, bypassing the GI tract, but it is increased when nutrients are absorbed through the GI tract. This suggests that the response of the mammary gland to nutrient status may involve the recognition of nutrient input by the GI tract and signaling of this input to the mammary gland. Such signaling pathways have not been described in lactating ruminants but would be expected to involve neural mechanisms, because severance of the vagal innervation to the GI tract of the lactating rat reduces milk yield (Eriksson *et al.*, 1994). This is associated with a reduction in the circulating concentrations of blood hormones such as PRL and oxytocin (OT). Changes in the release of these hormones due to an interrupted vagal innervation to the GI tract could be instrumental in the reduction in milk yield. In monogastric animals, neural pathways, in particular the vagus nerve, have been implicated in the regulation of nutrient homeostasis as well as homeorhesis (Eriksson *et al.*, 1994; Uvnas-Moberg, 1992; Uvnas-Moberg, 1989). These observations suggest a fundamental, but hitherto unrecognized, neural/endocrinal relationship may exist in ruminants, between the GI tract, brain and the mammary gland.

Therefore, this thesis explores in depth the neuroendocrine relationships between the GI tract, brain, pancreas and the mammary gland that are involved in insulin release and nutrient metabolism in a ruminant species, the sheep. The literature is reviewed in

relation to energy and amino acid partitioning and insulin release, with special reference to vagal nerve activity in the GI tract and its regulatory potential.

1.2 THE VAGAL INNERVATION OF THE GASTROINTESTINAL (GI) TRACT

1.2.1 Introduction

The vagus nerve is the longest and most widely distributed of the cranial nerves in vertebrates. The vagal neural network comprises left and right vagus nerves, nucleus tractus solitarius (NTS) and the dorsal motor nucleus (DMN) of the vagus. The two vagal nerves originate in the medulla oblongata of the brain stem where the NTS and DMN are located. The vagus nerve contains visceral sensory (afferent) and parasympathetic motor (efferent) fibres which, respectively, carry impulses from the viscera to the brain, and from the brain to the target organ. The NTS receives signals from the peripheral tissues in the neck, thorax and abdomen and conveys information to the DMN. The DMN, in turn, acts and sends the feed-back signal to the target organ and elicits the effect. Knowledge of the anatomy of the vagus nerves helps in understanding the physiological regulations associated with them. This section describes briefly the anatomy of the NTS and DMN together with the peripheral pathways of the left and right vagus nerves, and their innervation of the GI tract in ruminant species.

1.2.2 Nucleus Tractus Solitarius (NTS)

The NTS is the first synaptic site for vagal afferent fibres from the abdominal viscera, including the GI tract (Shapiro and Miselis, 1985; Kalia and Sullivan, 1982; Kalia and Mesulam, 1980). Although not completely distinguishable, there are visible nuclei formed by collections of different types of vagal neurons in the medulla oblongata. The NTS is a longitudinal collection of poorly myelinated or unmyelinated sensory nerve fibres of the vagus nerve. This first order afferent input terminal (NTS) receives afferent information from the stomach and duodenum (Norgren and Smith, 1988), liver (Rogers and Hermann, 1983), and perhaps the pancreas (Rinaman and Miselis, 1987). However, the receptive area for afferent inputs into the NTS may vary among species. For example, in rats, the medial area of the NTS receives gastrointestinal inputs, while the intermediate area receives respiratory tract and some airway inputs (Altschuler *et al*., 1989).

1.2.3 Dorsal Motor Nucleus of the vagus (DMN)

The DMN is a collection of efferent neurons that carries information away from the higher centres to the target organ (e.g. pancreas). Efferents from the DMN supply the preganglionic parasympathetic innervation to the organs of the thorax and abdomen. Like the NTS, functional organotopic separation within the DMN can be seen in some species (e.g. the pigeon and rat), however, the degree to which this separation occurs is not consistent across all species (Kalia and Mesulam, 1980). Getz and Sirnes (1949) have shown that the efferent fibres arising from the cranial and central portions of the DMN of the vagus innervate the GI tract.

1.2.4 Interconnections between the NTS and DMN of the vagus

The interconnection between the NTS and DMN of the vagus is important since reflex responses are carried to the peripheral target organ through the efferents of the DMN of the vagus. The demonstration that the dendrites of the gastric motor neurons in the DMN were labelled when the anterograde tracer, Cholera toxin horseradish peroxidase (CT-HRP) was injected into the stomach wall of the rat, suggests that NTS and DMN of the vagus are well interconnected (Shapiro and Miselis, 1985). Shapiro and Miselis (1985) have shown that dendritic processes from the DMN neurons extend well beyond the confines of the nucleus into the NTS. That the vagal afferents and efferents from the NTS and DMN of the vagus, respectively, form synapses at this level, provides the potential to co-ordinate information between peripheral organs and the brain.

1.2.5 Peripheral pathways of the vagus nerve in ruminants

The left and right vagus nerves originate in the medulla oblongata and descend along the dorsal aspect of the common carotid artery. After innervating the heart and lungs, the left and right vagus nerves anastomose cranial to the diaphragm and then divide to form the ventral and dorsal vagus nerves. The ventral and dorsal vagus nerves enter the abdominal cavity where they innervate the GI tract and its associated structures such as the liver and pancreas. These two nerves, like the cervical and thoracic vagus, contain both afferent and parasympathetic efferent fibres (Evans and Murray, 1954).

1.2.5.1 Ventral vagus nerve

The early findings by Irving *et al.* (1937) show that the stomach, pylorus and duodenum receive afferent fibres from the ventral vagus nerve (ventral vagal trunk). The ventral vagus nerve sends branches to the liver (hepatic branch), duodenum and pylorus (long pyloric branch). The nerve then continues ventrally in the lesser omentum giving branches to the reticulum, omasum, and ending at the abomasum (Habel, 1956). Figure 1.1 shows the innervation of the GI tract from the ventral vagus nerve.

1.2.5.2 Dorsal vagus nerve

The dorsal vagus nerve (dorsal vagal trunk) mainly supplies the rumen. It also innervates the reticulum and omasum before it runs over the visceral side of the lesser curvature of the abomasum, to the pylorus, which it also supplies. Figure 1.2 shows the innervation of the GI tract from the dorsal vagus nerve.

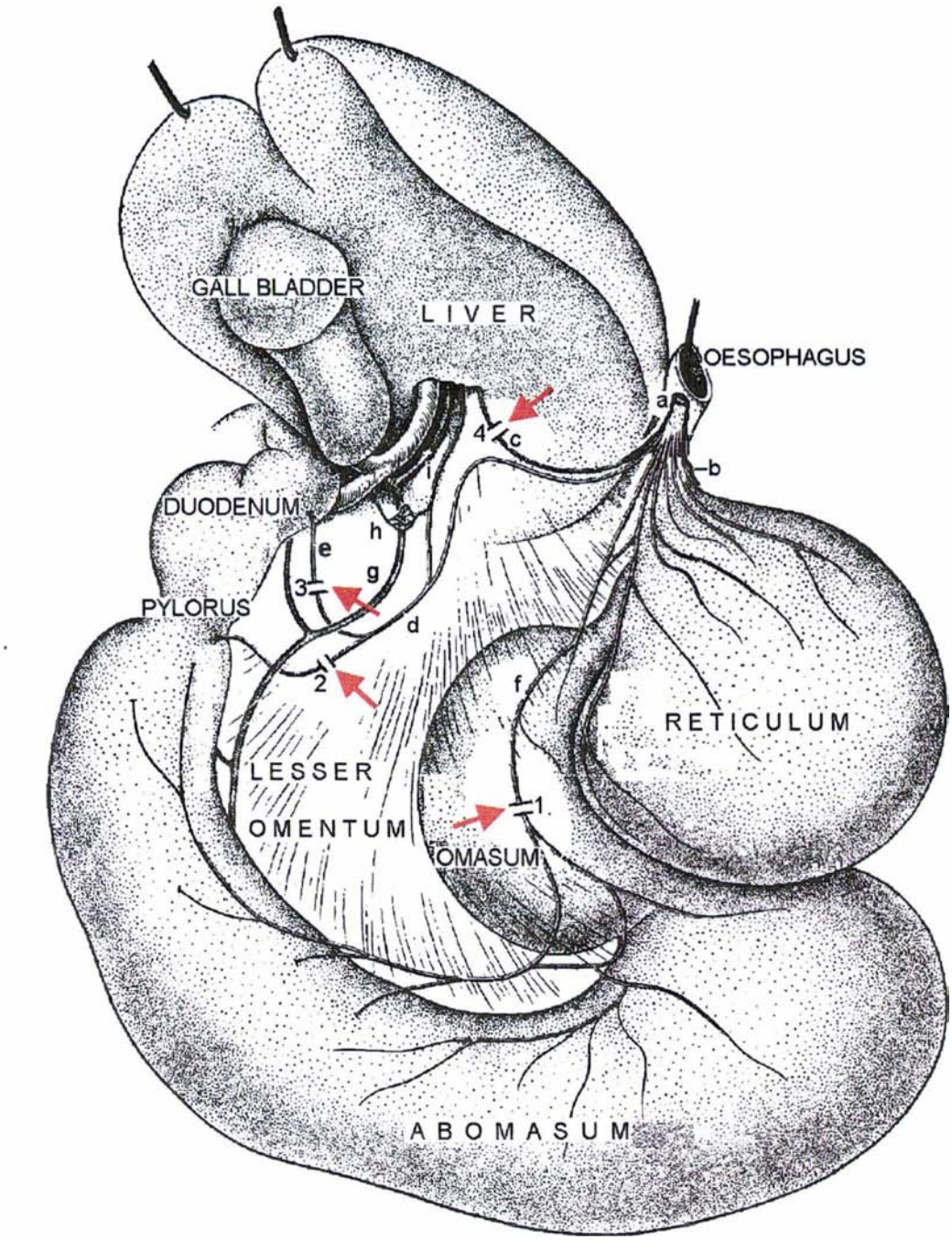

Figure 1.1 Ventral vagal innervation of the forestomach, upper small intestine and liver of sheep, right cranial aspect. The oesophagus and liver are raised. Abbreviations:*a*, ventral vagus nerve; *b*, branch to left side of atrium ruminis; *c*, hepatic branch; *d*, long pyloric branch; *e*, duodenal branch; *f*, continuation of ventral vagus nerve; *g*, right gastric artery; *h*, sympathetic plexus along right gastric artery; *i*, left branch of hepatic artery. Arrows indicate denervation sites (numbered as 1, 2, 3 & 4); for details of denervation (vagotomy) see Sections 2.3.2.1 and 5.3.2. (Diagram reproduced from Habel, 1956).

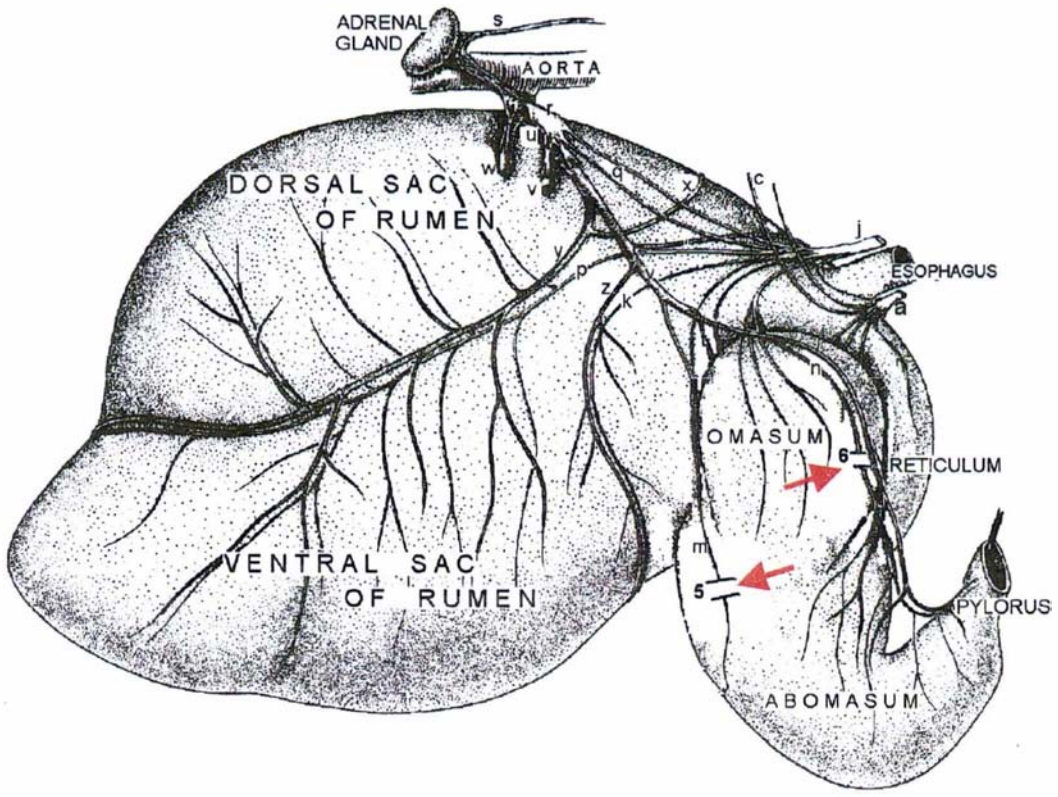

Figure 1.2 Dorsal vagal innervation of the forestomach of sheep, right aspect. The omasum and abomasum have been turned forward. *j*, dorsal vagus nerve; *k*, branch to plexus on left ruminal artery; *m*, abomasal branch; *n*, continuation of dorsal vagus nerve; *p*, right ruminal branch; *q*, branches to coeliac plexus; *r*, coeliacomesenteric ganglion; *s*, greater splanchnic nerve; *t*, left gastroepiploic artery; *u*, coeliac artery; *v*, hepatic artery; *w*, cranial mesenteric artery; *x*, splenic artery; *y*, right ruminal artery; *z*, left ruminal artery; *a*, ventral vagus nerve; *c*, hepatic branch. Arrows indicate denervation sites (numbered as 5 & 6); for details of denervation (vagotomy) see Section 3.3.2. (Diagram reproduced from Habel, 1956).

1.2.5.3 Comparison of the vagal innervation of the GI tract of the ruminant and the rat

The general distribution of the vagus nerve and its innervation of the stomach and upper small intestine of the GI tract in ruminants is similar to that in rats. In the rat, after anastomoses between the left and right vagus nerves, the ventral vagus nerve passes from the ventral aspects of the oesophagus to the lesser curvature of the stomach. Branches of the ventral vagus nerve include the hepatic, left gastric, coeliac and accessory coeliac nerves.

The dorsal vagus nerve penetrates the diaphragm dorsal to the oesophagus. It gives off the right gastric and coeliac branches (Powley *et al.*, 1983). Although innervation of the pancreas by a branch of the hepatic nerve from the ventral vagus has been described in rats (Niijima *et al.*, 1990; Yamazaki and Sakaguchi, 1989), such innervation is, however, not clear in ruminants. Anatomical studies using anterograde and retrograde tracing methods have shown that in the rat, vagal efferent axons run distally from the stomach to the intestine and selectively terminate on command neurons, which activate intrinsic enteric neurons of the myenteric plexus (Kirchgessner and Gershon, 1989). Many of these enteric neurons enter the pancreas, forming an extensive entero-pancreatic innervation (Kirchgessner and Gershon, 1990; Poulsen *et al.*, 1983).

The main difference between ruminant and monogastric animals from the physiological point of view is that in the former, the integrity of the vagus is essential for the transport of ingesta from the reticulum to the abomasum, eructation, regurgitation and ruminoreticular contraction cycles (Gregory, 1982; Harding and Leek, 1971; Stavney *et al.*, 1963; Andersson *et al.*, 1959; Iggo, 1956; Duncan, 1953; Dougherty and Habel, 1955). So that apart from vagally mediated mechanisms involved in metabolism, the vagus in ruminants has an essential role in the control of stomach activities.

1.2.6 Neural relationship between the hypothalamus, mammary gland, GI tract and the pancreas

Anatomical and electrophysiological studies provide evidence for a neuroendocrine axis between the hypothalamus, mammary gland and GI tract and also its associated organs such as the pancreas. Figure 1.3 shows a simple schematic

representation of this axis in mammalian species. The vagus nerve innervates the GI tract (Habel, 1956). Parasympathetic efferent fibres of the gastric branches of the vagus nerve have been found to communicate with enteric neurons, which, in turn, form an extensive enteropancreatic neural network (Kirchgessner and Gershon, 1989, 1990; Poulsen *et al.*, 1983). At the hypothalamus level, the paraventricular nucleus (PVN) and supra optic nucleus (SON) receive information from the mammary gland, carried through sensory fibres of the genitofemoral nerve (Tindal, 1978; Tindal and Knaggs, 1975). These two OT producing nuclei exchange information with the two vagal nuclei, NTS and DMN (Siaud *et al.*, 1989; Rogers and Hermann, 1986, 1985; Sims and Lorden, 1986; Van der Kooy *et al.*, 1984; Sawchenko and Swanson, 1982; Saper *et al.*, 1976). The reflexes mediating the effects of sight, sound, smell or taste, for example, on cephalic phase insulin release and conditioned reflex release of OT, all depend upon the integrity of the vagus nerve (Niijima, 1991; Niijima *et al.*, 1990; Strubbe and Steffens, 1975). These findings indicate that the vagus nerve plays a significant role in coordinating the peripheral organs with the brain.

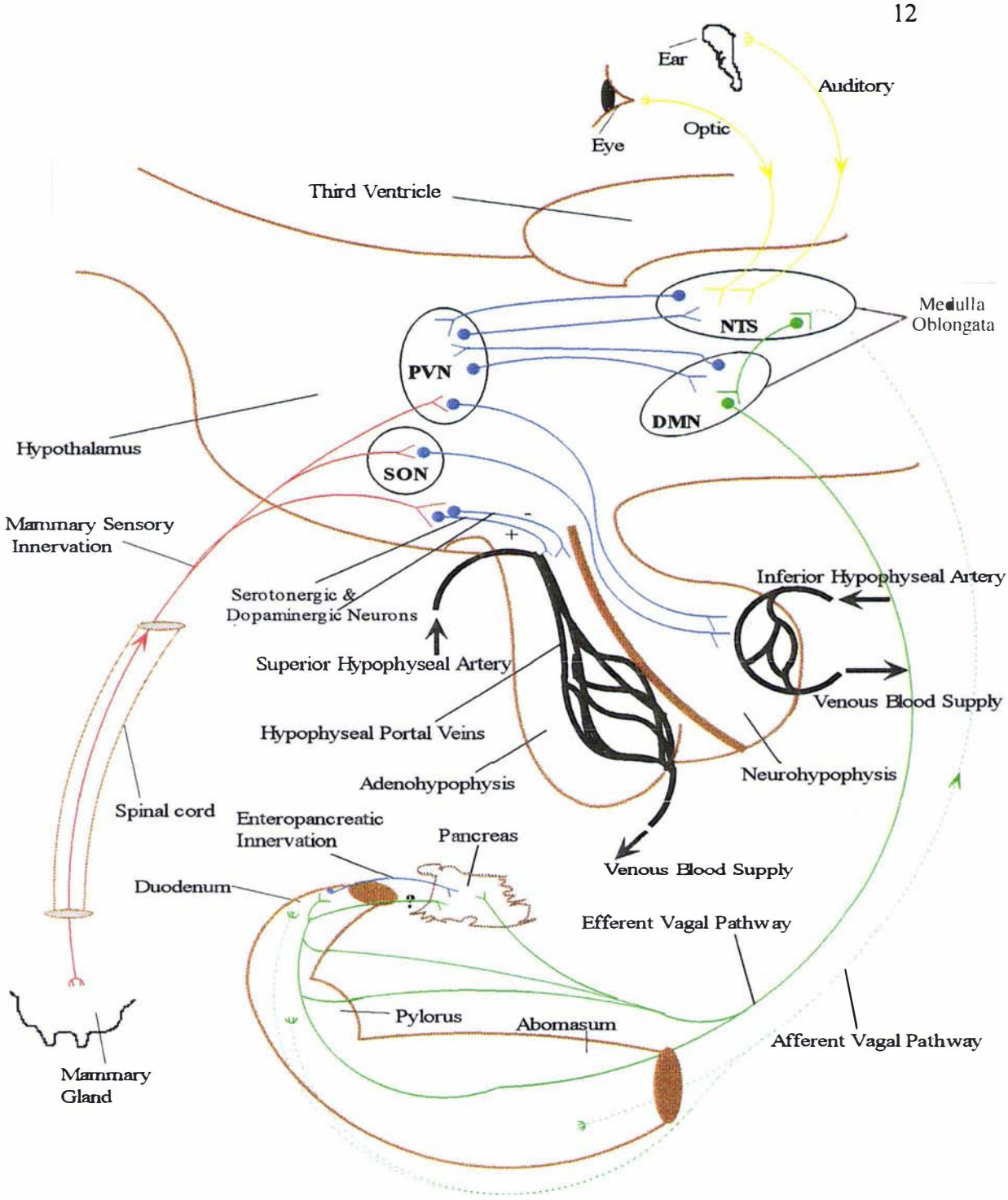

Figure 1.3 A simple schematic representation of the neuroendocrine relationship between the hypothalamus, mammary gland, gastrointestinal tract and the pancreas in a mammalian species. The vagal afferent (----), vagal efferent (—), mammary sensory (—), cephalic ( ) pathways and their communication with other neuroendocrine components in the hypothalamus and GI tract (—) are shown. The arrow of the sensory pathways indicates the direction of signal. Abbreviations: PVN, paraventricular nucleus; SON, supra optic nucleus; NTS, nucleus tractus solitarius; DMN, dorsal motor nucleus of the vagus nerve.

1.2.7 Summary

The vagal network formed by various neuronal components such as afferent and efferent vagal fibres, NTS, and DMN, co-ordinates signals between the brain and peripheral organs in the body. The NTS receives signals from the peripheral organs via the afferents and these inputs are conveyed to the DMN. The DMN, in turn, replies to the target organ via the vagal efferents and allows the target organ to exert the function. For example, vagal afferent fibres stimulated by nutrients in the GI tract carry information about the availability of food to the brain and this is followed by a feed-back response from the brain to the target organs such as the pancreas and GI tract for endocrine secretion, and other GI tract activities. In addition, the midbrain nuclei associated with the vagus nerve appear to play a role as relaying centres for reflex responses.

1.3 NUTRIENT SUPPLY AND METABOLISM IN LACTATING RUMINANTS

1.3.1 Introduction

The increased demand for nutrients by the mammary gland during lactation, particularly in domesticated ruminants bred for dairy production, is met by various strategies that are adapted in a well-coordinated fashion during this phase of the reproductive cycle. These regulatory mechanisms drive a substantial proportion of the nutrients either absorbed from GI tract or released from body reserves, to the active mammary gland. This is the basis of the concept of homeorhesis in which nutrients are preferentially partitioned towards those body tissues which require a higher priority for nutrients such as the mammary gland for milk synthesis (Bauman and Currie, 1980; Bauman and Elliot, 1983).

All these homeorhetic changes, together with alterations in the relative amplitude of the homeostatic control mechanisms, enable the animal to remain in physiological equilibrium despite increased nutritional and metabolic demands created by the lactating mammary gland. Clearly greater demand for nutrients by the lactating mammary gland creates diverse changes in whole body metabolism. The term "nutrient partitioning" is widely used to describe the manner in which absorbed nutrients are diverted towards those body tissues and organs that have a higher priority for nutrients. However, it is equally

important to consider the changes in the food intake and GI tract that are directly associated with physiological processes such as lactation. These changes enhance the availability of nutrients for absorption and thus facilitate nutrient partitioning.

1.3.2 Effect of lactation on voluntary food intake

Food intake is markedly enhanced during lactation due to a large demand for nutrients and the female of many species can eat enough during lactation to support milk synthesis without having to call to any great extent on their body reserves, as long as good quality food is available *ad libitum* (Dial and Avery, 1991; Singh and Singh, 1990; Sainz *et al.*, 1986; Jansen and Hunsaker, 1986; Roberts and Coward, 1985; Cripps and Williams, 1975; Leshner *et al.*, 1972; Forbes, 1970; Campling, 1966; Anderson and Turner, 1963; Fell *et al.*, 1963). For example, during lactation, food consumption in rats increases 2-3 times compared with that of non-lactating, non-pregnant animals, and returns to non-lactating intakes within 9 days of weaning (Anderson and Turner, 1963). After a slight fall in feed intake immediately following parturition, a rapid increase in food intake takes place between day 3 and day 18 of lactation in rats (Cripps and Williams, 1975).

Nutrient demand for milk synthesis during lactation in dairy breeds is even higher because they have been selected for high milk production (Marston and Lusby, 1995). Because of this demand food intake increases, although the increases in intake fails to keep pace with the increase in energy expenditure, which leads to an increased mobilisation of body reserves (Wilson, 1983; Fell *et al.*, 1972b).

1.3.3 Lactation-associated changes in the GI tract

Development of the GI tract during lactation is necessary so that it is able to digest the increased amounts of food and absorb the nutrients released. Lactation associated changes in the GI tract have been described by various authors (Weekes, 1972; Fell *et al.*, 1972a; Crean and Rumsey, 1971; Tulloh, 1966a, 1966b; Fell *et al.*, 1964).

Crean and Rumsey (1971) observed that the gastric mucosa was hypertrophied in lactating rats. Fat-trimmed weights of each part of the GI tract were larger in lactating than in dry cows (Tulloh, 1966b). Tulloh (1966a) found that in lactating cows the size of the rumen increased relative to the rest of the stomach. There was also hypertrophy of the

mucosa of both the stomach and the intestine. Hypertrophy of the abomasum, intestine, and rumen together with increasing weight of the rumen mucosa and emptied intestine has been reported during early lactation in lactating ewes (Fell *et al.*, 1972a; Fell *et al.*, 1964). Campbell and Fell (1970) also reported that changes in rumen weight were paralleled by changes in the weight of liver and increased liver enzyme activity.

1.3.4 Carbohydrate metabolism

It is well known that glucose is a key metabolite in energy metabolism and other cellular activities in mammals. Glucose is derived directly by intestinal absorption in non-ruminants whereas it is produced mainly in the liver and also the kidneys from gluconeogenic precursors via gluconeogenesis in ruminant species. Propionate, a major precursor for glucose synthesis in ruminants, is derived mainly from microbial degradation of dietary carbohydrates in the rumen. The liver removes most of the propionate in the portal blood so little enters the general circulation. The two other major VFA's derived from degradation of carbohydrates, namely butyrate and acetate, are not gluconeogenic. Butyrate is converted to β-hydroxybutyrate in the rumen epithelia and liver, and with acetate enters the general circulation and is used for energy and fat synthesis in the adipose tissue and mammary gland.

Glucose is the major precursor for the synthesis of lactose, the major osmole drawing water into milk. It is used for the synthesis of glycerol and also required for the production of NADPH for fatty acid synthesis in the mammary gland (Chaiyabutr *et al.*, 1980; Kuhn, 1983). The lactating mammary gland can use up to 85% of the total glucose turnover in the body, depending on the level of production (Bickerstaffe and Annison, 1974; Bergman *et al.*, 1974). Two thirds or more of the mammary uptake is converted to lactose. Because of this large demand for glucose for milk synthesis, utilization of glucose by other body tissues for energy is altered to allow partitioning of a greater percentage of glucose to the mammary gland. For example, total glucose oxidation to carbon dioxide is about 34% at day 30 prepartum, reducing to only 8-9% by day 7 of lactation (Bennink *et al.*, 1972).

Gluconeogenesis is the main process providing glucose to the mammary gland in ruminant species. In the dairy cow, gluconeogenesis occurs mainly in the liver, which

accounts for about 85% of glucose synthesis while the remainder is synthesized in the kidney. Amino acids, lactate, glycerol and in particular propionate are the main precursors for glucose synthesis (Bergman *et al.*, 1974). Alanine and glutamine are the major amino acid glucogenic precursors and account for about 40-60% of amino acids used for gluconeogenesis (Bergman and Heitmann, 1978). The activities of key gluconeogenic enzymes in the liver of lactating ruminants compared to non-lactating state, are also changed, leading to increases in the efficiency of conversion of various precursors into glucose (Mercier and Gaye, 1983). Hepatic extraction of lactate is also increased, facilitating gluconeogenesis in the liver (Baird *et al.*, 1980).

1.3.5 Protein metabolism

The turnover of proteins, the process of continuous synthesis and degradation of all proteins in all cells, is a homeostatically controlled mechanism. Up to 10% of all body protein is turned over each day while free amino acids represent only about 0.1% of the total body amino acids (Swick and Benevenga, 1977). This turnover of proteins provides a mechanism for a continuous redistribution of amino acids into proteins essential for life at that moment, for example milk synthesis. As for glucose, amino acid requirements for lactating mammary gland are also very high. The majority of amino acids absorbed from the GI tract, such as alanine, glycine, serine and glutamate, are removed by the liver and used as gluconeogenic precursors (Bergman and Heitmann, 1978). Milk protein synthesis in lactating ruminant mammary gland depends mainly on GI tract absorbed amino acids. However, the demand for amino acids for milk protein synthesis is met by both amino acids absorbed from the GI tract and degradation of body protein, although contribution from body proteins towards total milk protein synthesis is relatively minor (Peel and Bauman, 1987).

Amino acids can provide up to 12% of lactose in milk via gluconeogenesis (Hunter and Millson, 1964). The synthesis of milk protein in a dairy cow producing moderate quantities of milk (i.e. 22 kg per day) accounts for at least 20% of whole body protein synthesis (Oldham *et al.*, 1980). The increase in intake during early lactation often fails to keep pace with the increase in protein metabolism and a mobilisation of body proteins usually occurs. Thus, during the initial stages of lactation, when the intake of

energy and protein does not meet the amounts secreted in the milk, muscle protein synthesis is depressed below the normal rate of degradation and the amino acids released are channelled into milk protein production. This condition persists until such time that food intake increases or milk production decreases, bringing the animal back into equilibrium. This appears to be a normal metabolic adaptation to meet the challenge of the high rate of milk production. For example, Bryant and Smith (1982) have shown that during early lactation the protein mass of specific muscles in ewes was 25-40% less than for unmated or late lactation animals. It was also shown that nearly 25-27% of labile body protein is readily available to supply amino acids for supplementation of dietary intake of amino acids for milk protein synthesis during early lactation of the dairy cow (Botts *et al.*, 1979). However, the quantity of milk the body protein reserves could produce depends on factors such as availability of limiting amino acids for milk synthesis, efficiency of body protein utilization, and quantity of the reserve utilized for glucose synthesis (Swick and Benevenga, 1977).

1.3.6 Lipid metabolism

Adipose tissue is a vital organ in the body and is a dynamic energy store. About 30-35% of the flux of metabolizable energy intake passes through adipose tissue in the lactating dairy cow (Emery, 1979). Together the lactating mammary gland and the adipose tissue are the major sites for fatty acid synthesis in lactating ruminants. A major portion of milk fat and adipose tissue fat in dairy cows is derived from acetate because their dietary intake of long chain fatty acids is limited to about 2-5% of the diet (Emery, 1979).

Food intake does not usually match the demand for nutrients early in lactation and the net energy deficit occurs even with *ad libitum* feeding for high producing dairy animals (Flatt *et al.*, 1965). This results in a need for rapid mobilisation of stored lipids with concomitant decrease in the uptake of nutrients for lipid synthesis to meet the energy deficit. Thus, adipose triglycerides are hydrolysed to glycerol and free fatty acids and released into the blood (Emery, 1979). During this period, fat rather than glucose, is used as the predominant source of energy. This aids the flow of glucose towards the mammary gland for milk synthesis. The energy deficit in high yielding dairy cows during the first 10 weeks of lactation has been calculated to be energetically equivalent to approximately 50

kg of pure lipids or an average daily production of 9 kg milk. During the first month of lactation, it is equivalent energetically to about one-third of the milk produced (Bauman and Currie, 1980). Flatt *et al.* (1965) reported that in early lactation, a Holstein cow producing over 45 kg milk per day lost 16-20 Mcal energy per day. They further reported that the cow was near energy equilibrium during mid lactation when she was producing about 22-30 kg milk per day whereas the cow started to deposit energy (15-19 Mcal per day) during late lactation with milk production of 6-11 kg per day.

The various changes in adipose tissue during lactation include a shift from lipid synthesis to mobilisation in adipocytes. This change is facilitated by adaptations which include a decrease in lipoprotein lipase activity (Mendelson *et al.*, 1977; Hamosh *et al.*, 1970) and the activity of key enzymes in the regulation of *de novo* fatty acid synthesis (Chilliard *et al.*, 1977; Fain and Scow, 1966). The net effect is that lipid synthesis or energy storage in adipose tissue is diminished (Shirley *et al.*, 1973; Sidhu and Emery, 1972) while rates of lipolysis are increased (Metz and Bergh, 1977; Sidhu and Emery, 1972), leading to an increased concentrations of NEFA in the circulation. Conversely, in the mammary gland, an increased activity of lipoprotein lipase is necessary to make available fatty acids and glycerol from triglycerides, transported in chylomicrons and very low density lipoproteins (Mendelson *et al.*, 1977; Hamosh *et al.*, 1970). Hamosh *et al.* (1970) showed that lipoprotein lipase activity in the mammary tissue of the rat increased 1 to 2 days before parturition and remained elevated throughout lactation. Furthermore the activity of the enzyme increased in response to suckling whereas it was decreased by 70% within 9 h and disappeared completely within 18 h when suckling was stopped. This kind of adaptation also ensures the increased availability of NEFA for the synthesis of milk fat.

1.4 NUTRIENT PARTITIONING DURING LACTATION

Nutrient partitioning is a complex process and the regulation of which involves a variety of central and peripheral mechanisms. Since food intake is closely associated with nutrient partitioning, regulation of food intake is necessary. Subsequently, during lactation, regulation of nutrient partitioning is important in order to efficiently partition nutrients between the mammary and extramammary tissues (Bauman and Currie, 1980).

1.4.1 Control of food intake

Regulation of food intake involves a variety of signals, many of which are either metabolic, hormonal or neuro-humoral in origin. In addition, signals are transmitted to the CNS via the nerves from mechano- and chemoreceptors in the GI tract (Forbes and Barrio, 1992). Mayer (1953) suggested that voluntary food intake is controlled by blood metabolites. Forbes (1988) suggested that the levels of many blood metabolites such as glucose, fatty acids and amino acids are involved in the control of food intake in mammals. Intraintestinal infusion of amino acids and fatty acids (Yox *et al.*, 1991) and i.v. administration of fatty acids (Wirtshafter and Davis, 1977) or glucose (Campfield and Smith, 1986) caused satiety in rats.

The liver is the principle organ involved in the post-absorptive metabolism of nutrients and is the first of the splanchnic organs to store nutrients in the post-absorptive period. Russek (1963) proposed that the liver is central to the metabolic control of food intake in monogastric animals. However, the mechanism by which nutrients administered into the systemic circulation or GI tract, exert satiety is not fully understood.

Russek (1963) proposed the existence of a hepatostatic mechanism by which glucose regulates feeding in monogastric animals. This hypothesis was based on observations that i.p. injection of glucose caused satiety in dogs. The author also observed that the hyperglycaemia which occurred 5 min after the injection coincided with maximum anorectic effect. Portal infusion of glucose in rats reduces food intake (Tordoff *et al.*, 1989). Niijima (1983, 1982) demonstrated that glucose sensitive afferent vagal fibres are present in the portal area and in the liver. Furthermore this hepatic gluco-sensitive pathway reaches the hypothalamus of the rat, in that hepatic portal glucose infusion strongly affected excitatory activity of many cells in the lateral hypothalamus (Shimizu *et al.*, 1983). Others (Novin, 1983; Sawchenko and Friedman, 1979) have reviewed the involvement of the liver in the control of food intake in monogastric animals and concluded that the liver plays a major role in the initiation of satiety.

It has been suggested that the PVN of the hypothalamus is closely linked to the regulation of food intake, following the observation that catecholamines directly injected into PVN elicited feeding and that this effect was dependent upon an intact vagus nerve

(Liebowitz, 1980). A strong connection between PVN and DMN of the vagus was also reported (Ricardo and Koh, 1978). Thus, overall these data provide strong evidence that the vagal afferent innervation of receptors sensitive to glucose and perhaps other nutrients in both the liver and GI tract are likely to be involved in the control of food intake in monogastric animals.

Unlike monogastric animals, ruminants do not seem to be responsive to glucose infusions, but a role for fatty acids in the control of food intake has been reported and reviewed by Baile and Forbes (1974) and De Jong (1986). Duodenal infusion of peanut oil can depress intake in sheep (Titchen *et al.*, 1966). Although Thye *et al.* (1970) reported that a positive correlation exists between plasma levels of total fatty acids and the amount of food eaten in sheep, long-term i.v. infusion of long-chain fatty acids, for example palmitic, stearate and oleic acid, depressed food intake in sheep (Vandermeerschen-Doize and Paquay, 1984).

Although comparatively few studies of the effect of VFA on food intake have been made, available evidence suggests that VFA can reduce food intake in ruminants when administered intra-portally or intraruminally (Farningham and Whyte, 1993; Baile and Forbes, 1974; Baile and Mayer, 1968). Infusion of propionate into the hepatic portal vein in physiological amounts depressed feeding in sheep (Farningham, 1990; Anil and Forbes, 1988, 1980). The reduction in intake was dose-related (Farningham and Whyte, 1993) and it was significant on both 3 h as well as 24 h food intake in response to a 3 h infusion period.

The mechanism by which intraruminal or i.p. infusions of VFA depress food intake is not understood. However, it is possible that in ruminants, like monogastric animals whose gluco-receptors in the portal area and GI tract are in close connection with the afferent vagal innervation (Novin, 1983), that propionate may signal food intake control centres in the brain through vagal afferents innervating liver/portal receptors. Indeed, a complete denervation of the liver abolished the depressing effect on food intake in response to i.p. administration of propionate in sheep (Anil and Forbes, 1980). This indicates a neural pathway from the liver to the brain informing the latter of the metabolic status of the liver. Later Anil and Forbes (1988) confirmed these results by demonstrating

that hepatic vagotomy in sheep abolished the depressing effect of portally infused propionate. The satiety induced by propionate is probably mediated through propionate receptors and not through glucose receptors (Niijima, 1983, 1982), because glucose does not have any effect on food intake in ruminants (Baile and Forbes, 1974; Peterson *et al.*, 1972; Baile *et al.*, 1970; Baile and Mahoney, 1967; Holder, 1963; Dowden and Jacobsen, 1960; Manning *et al.*, 1959).

Anil and Forbes (1988) also observed that when sympathetic splanchnic branches were sectioned the satiety effect of i.p. propionate infusion was reduced, suggesting that splanchnic afferents may also play a role in this control mechanism in sheep. This led to the conclusion that regulation of intake may involve several different mechanisms that are integrated at the brain stem level (Norgren, 1983). However, anorectic effects of metabolites such as glycerol, lactate and pyruvate were found to be dependent on the integrity of the vagus nerve (Langhans *et al.*, 1985). Furthermore an increased release of ATP in liver cells following oxidation of substrates modulates sodium pump activity and membrane potential which are inversely related to vagal discharge rate (Niijima, 1983, 1982). Taken together, all these results suggest that the integrity of the vagus nerve is probably the most important factor in the integrated food intake control mechanisms in ruminants.

Insulin has been shown to cause satiety in animals. Secretion of insulin occurs at the onset of eating and can also be released by the sight of the food in monogastric animals (Berthoud *et al.*, 1980, 1981) and ruminants (Faverdin, 1986a; Vasilatos and Wangsness, 1980; Bassett, 1975; Bassett, 1974a; Lofgren and Warner, 1972). An i.v. injection of insulin decreased sham feeding in rats feeding with open gastric cannulas (Oetting and Vanderweele, 1985). This agrees with the findings of Nicolaidis and Rowland (1976) who showed that in rats, the addition of a small amount of insulin to an i.v. glucose infusion caused a greater reduction of food intake than the glucose alone. Although similar effects of i.v. insulin on satiety in ruminants has been reported (Faverdin, 1986a; Baile and Martin, 1971; Houpt, 1974; Reid *et al.*, 1963), a definite role in the mediation of satiety is not clear. Insulin given intravenously at the beginning of a meal depressed food intake during the following 30 min in cows (Faverdin, 1986a). Baile and Martin (1971) reported that, in sheep, intake was reduced by 70% following i.v. administration of insulin. In

contrast, a study by Houpt (1974) has shown that exogenous insulin can increase intake in sheep. It was also evident that in alloxan-induced diabetes, inappetence and death were the main responses in sheep and that the intake levels were restored by replacement therapy with insulin (Reid *et al.*, 1963). It seems that the effects of systemic administration of insulin is variable and sometimes depresses food intake and sometimes stimulates it and the effect is probably influenced by glucose status.

However, it has been reported that insulin administered directly into the brain can depress food intake. Chronic infusion of insulin for 2-3 weeks into the lateral ventricles of the brain in baboons caused a dose-dependent reduction in food intake and body weight (Woods *et al.*, 1979). In rats, i.c.v. administered insulin reduced food intake (Plata-Salaman and Oomura, 1986; Hatfield *et al.*, 1974) while administration of insulin antibodies resulted in increased food intake and body weight (Strubbe and Mein, 1977; McGowan *et al.*, 1992). McGowan *et al.* (1992) reported that the sites within the ventromedial hypothalamic area most sensitive to the effects of insulin on food intake included the PVN and the nuclei of ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH). Interestingly, these 3 nuclei, the VMH, DMH and PVN, are associated with the vagus nerve (Yoshimatsu *et al.*, 1984). Yoshimatsu *et al.* (1984) showed that lesions produced in these nuclei increased vagal nerve activity in the rat. In line with this, it is also possible that insulin secreted after a meal acts on hypothalamic nuclei to affect food intake. Systemic administration of glucose affects the activity of neurons in the VMH (Anand *et al.*, 1964). Intravenous injection of insulin suppresses the increased activity of taste response neurons in the NTS in response to lingual application of 1.0 M glucose solution in rats (Giza *et al.*, 1992). Taken together, all these results indicate that insulin influences food intake, and the effect is likely to be mediated through the vagal efferent pathway connecting the brain and GI tract.

Glucagon is increased in blood during feeding and its effects on satiety have been extensively reviewed by Geary (1990). Food intake was strongly suppressed in response to i.p. (Geary and Smith, 1982) or i.c.v. (Inokuchi *et al.*, 1984) administration of pancreatic glucagon in rats. Lesions produced in the NTS in rats impaired the glucagon-induced suppression of food intake (Weatherford and Ritter, 1988). It was also shown that i.p. infusion of glucagon was completely ineffective in suppressing feeding in rabbits that were

vagotomized bilaterally whereas glucagon suppressed food intake in free feeding control rabbits (Vanderweele *et al.*, 1979). Together, these data indicate the involvement of vagal afferent and efferent pathways in glucagon-induced postprandial satiety in monogastric animals.

Glucagon-like peptide-1 (GLP-1), a hormone homologous to pancreatic glucagon, is produced in the GI tract during feeding (Faulkner and Martin, 1997). The possible role of this hormone in postprandial satiety in animals has been reviewed by Gunn *et al.* (1997). The i.v. administration of GLP-1 (7-36) enhances satiety in man (Flint *et al.*, 1998) and i.c.v. administration of GLP-1 strongly reduced food intake in rats (Turton *et al.*, 1996). This effect may be mediated through the specific GLP-1 receptors that are present in a number of hypothalamic nuclei (Christensen *et al.*, 1996). It was found that GLP-1 is also produced in the hypothalamic neurons and, the NTS is the primary site that contains GLP-1 producing neurons in rats (Larsen *et al.*, 1997). The i.c.v. administration of GLP-1 into PVN suppressed food intake in rats in proportion to the dose, suggesting that GLP-1 may act, in part, to suppress feeding through interactions with cells within the PVN (McMahon and Wellman, 1997). A close relationship between the NTS and PVN and GLP-1 suggests that the vagal pathway is probably the primary pathway involved in the GLP-1 induced satiety in these animals.

Cholecystokinin is a peptide present in high concentrations in the GI tract and brain in many species and its secretion is increased during feeding and subsequently as digesta passes through the stomach and duodenum. It suppresses food intake in monogastric animals and its role in control of food intake has been extensively reviewed (Baile *et al.*, 1986; Dockray, 1982; Morley, 1982).

Cholecystokinin suppresses intake while CCK receptor antagonists increase food intake when administered either centrally or peripherally (Maddison, 1977; Nemeroff *et al.*, 1978; Telegdy *et al.*, 1984; Pekas and Trout, 1993; Moran *et al.*, 1993; McLaughlin *et al.*, 1985; Smith *et al.*, 1981; Crawley and Beinfeld, 1983; Moran and McHugh, 1982). The vagal mediation of satiety induced by CCK is the generally accepted hypothesis for its mode of action (Reidelberger and O'Rourke, 1989; Shillabeer and Davison, 1985). Vagotomy eliminates the CCK-induced satiety effect in rats (Smith *et al.*, 1985; Lorenz

and Goldman, 1982; Smith *et al.*, 1981) and in broiler chickens (Covasa and Forbes, 1994). Conversely, if endogenous CCK, like exogenous CCK, acts through an abdominal vagus-mediated mechanism to produce satiety, then subdiaphragmatic vagotomy should block the stimulatory effect of CCK antagonists on feeding.

Indeed Smith *et al.* (1985) and Shillabeer and Davison (1985) have shown that the increased food intake induced by the CCK antagonist proglumide, is eliminated by abdominal vagotomy in rats. Gastric vagotomy in rats prevents the inhibitory effects of exogenous CCK on intake, which suggests that receptors for CCK that are involved in the feeding response are also present in the stomach (Smith *et al.*, 1981). Since the satiety signals induced by injection of CCK are transmitted via the vagus nerve, it is important to identify the active parts of the vagal system that are involved in the CCK-induced satiety. Thus, Crawley *et al.* (1984) traced the sensory pathway mediating the actions of peripheral CCK from GI tract to brain by producing selective bilateral lesions at the levels of the abdominal vagus, NTS, midbrain, and PVN in rats. In summary, at each level of the vagal system, satiety induced by peripheral injection of CCK is abolished by lesions produced bilaterally, suggesting that CCK released from the GI tract acts on peripheral receptors, which send information through the vagus nerve to the brain. Furthermore these results suggest that the effect of endogenous CCK is similarly dependent on the integrity of the vagus nerve in rats and perhaps in other monogastric animals. Although strong evidence is accumulating that the effect of CCK in initiating satiety is mediated through vagal CCK binding sites in monogastric animals (Mercer *et al.*, 1993, 1992; Moran *et al.*, 1990, 1987; Crawley *et al.*, 1984; Zarbin *et al.*, 1981), the exact mechanism by which CCK modulates food intake in ruminants has yet to be identified (Farningham *et al.*, 1993).

Although the satiety effect of bombesin, a polypeptide secreted from nerve endings in the GI tract, has been studied (Ladenheim and Ritter, 1989, 1988; Flynn, 1989), its role in the control of intake is not well established, unlike that for CCK.

Leptin is a peptide secreted by the adipose tissue (Maffei *et al.*, 1995). Available evidence suggests that leptin plays a major role in the control of adipose tissue mass by controlling food intake in rodents (Considine *et al.*, 1995), and the topic is extensively

reviewed by Friedman (1998). An increase in body fat leads to a rise in circulating leptin concentrations, which, in turn, decreases food intake and increases energy expenditure (Halaas *et al.*, 1997). Conversely, a loss of body fat results in decreased concentrations of circulating leptin, leading to an increase in food intake (Ahima *et al.*, 1996). Available evidence indicates that the primary site of action of leptin is the hypothalamus. Intracerebroventricular administration of leptin strongly suppresses food intake in mice (Campfield *et al.*, 1995; Halaas *et al.*, 1997). However, the efferent pathway for the regulation of food intake from the brain feeding centres induced by the action of leptin is unknown. Further studies indicated that neuropeptide Y and melanocyte stimulating hormone are involved in the hypothalamic responses to leptin at times of low (i.e. during starvation) and high (i.e. obese) circulating concentrations, respectively, (Campfield *et al.*, 1995; Erickson *et al.*, 1996). However, the precise mechanism(s) whereby leptin regulates food intake remains to be defined.

In summary, many peripheral signals either metabolic or hormonal in origin, appear to be integrated in the control of food intake. Most such signals are currently assumed to be transmitted from sensors in the GI tract and liver to the hypothalamus via the vagus nerve. Many signals that include variations in the concentration of nutrients or hormones resulting from ingestion of food are assumed to play important roles in controlling food intake in monogastric animals, although the mechanism(s) by which these signals cause satiety in ruminants are not clear. However, it is considered that the vagal innervation of the GI tract is also likely to play a similar role in the control of satiety in ruminants.

1.4.2 Hormonal regulation of nutrient metabolism

It is believed that hormones essential for the onset and maintenance of lactation, may also be involved in the control of nutrient partitioning in lactating ruminants. In this context, hormones released from the pituitary gland, adrenal glands and the GI tract and its accessory organs such as the pancreas, play an integrated role and are discussed in this section.

1.4.2.1 Insulin

Blood glucose concentrations are generally maintained relatively constant by insulin, a peptide secreted from the β-cells of the pancreas. Insulin stimulates the uptake of glucose by various tissues and their utilization within the cells, thus reducing blood glucose concentrations. It stimulates the muscle uptake of glucose and its conversion to glycogen. Insulin inhibits gluconeogenesis in the liver and kidneys and reduces glucose release into the general circulation (Brockman, 1978).

Regulation of glucose metabolism in the lactating ruminant is very important since the mammary gland has a large demand for glucose for the synthesis and secretion of milk solids, in particular lactose. Much less is known about insulin action during established lactation in ruminants. Plasma insulin concentrations were lower in high yielding than in low yielding cows (Hart *et al.*, 1978) and in early lactation, insulin concentrations were lower than that in non-lactating cows (Sartin *et al.*, 1988), although it is difficult to interpret these differences that may reflect differences in milk yield or differences in net energy balance. The release of insulin from the pancreas is less responsive to increasing concentrations of blood glucose in lactating cows (Lomax *et al.*, 1979). Insulin secretory response to glucose infusion was also lower during lactation than in the heifer or during the dry period in dairy cows (Sartin *et al.*, 1985b).

Insulin plays a crucial role in regulating the supply of substrates to the mammary gland by altering peripheral extramammary metabolism (Metcalf *et al.*, 1991; Tesseraud *et al.*, 1992). Although insulin is required for mammary growth and maintenance of normal mammary functions, it is believed that insulin has no direct effect on glucose uptake by the ruminant mammary gland. Insulin-induced hypoglycaemia results in a decrease in lactose yield (Linzell, 1967), but simultaneous continuous infusion of glucose fails to alter mammary uptake of glucose and lactose secretion in lactating goats (Tesseraud *et al.*,1992; Linzell, 1967; Hove, 1978a, 1978b) or cows (Laarveld *et al.*, 1981). This agrees with findings of Neville *et al.* (1993) who showed in lactating women that a hyperglycaemic-euglycaemic clamp failed to increase lactose synthesis and milk secretion rate, even though milk glucose concentration increased three to four fold. They also noted that when plasma insulin concentration increased to about ten times baseline concentration

for 4 h it had no effect on lactose synthesis, milk volume or milk glucose concentration. Moreover in support of the short term glucose clamps, insulin and glucose infused for longer periods of time (i.e. for 4 days) have also failed to alter milk yield in cows (Griinari *et al.*, 1997a, 1997b; McGuire *et al.*, 1995).

During lactation, peripheral tissues are also less sensitive to insulin so that substrates for milk synthesis are not so readily taken up from the circulation. As a result, concentrations of many milk precursors are increased in the circulation. Thus, the increased availability of nutrients allows constant and continuous flow of nutrients towards the mammary gland, in spite of fluctuating glucose flow to other body tissues in response to changes in plasma insulin concentrations (Bauman and Currie, 1980). This kind of adaptation makes it possible for the lactating mammary gland to use up to 85% of the total glucose turnover in the body, depending on the level of production and thereby maintain milk synthesis and secretion in dairy cows (Bickerstaffe and Annison, 1974).

Insulin stimulates protein synthesis and amino acid uptake by peripheral tissues, although, as stated earlier, peripheral tissues and the mammary gland in lactating ruminants are less responsive to circulating insulin (Lomax *et al.*, 1979). Insulin infusion for 4 h with simultaneous amino acid infusion failed to increase amino acid uptake by goat (Tesseraud *et al.*, 1992) or bovine (Metcalf *et al.*, 1991; Laarveld *et al.*, 1981) mammary gland. However, Griinari *et al.* (1997a) reported that abomasal infusion of casein combined with the insulin clamp increased milk and protein yields. Milk protein yield was 10% greater than baseline values when casein was infused alone, but increased by 28% when combined with insulin clamp (Griinari *et al.*, 1997a). These observations, however, suggest that insulin does alter mammary metabolism in lactating ruminants. The discrepancy in the results between short- and long-term infusion studies is probably due to the duration of infusion times. However, the stimulatory effects of insulin on protein and milk yield require further investigation in lactating ruminants.

Insulin plays a major role in fat metabolism in ruminants. It increases lipogenesis and decreases lipolysis in the adipose tissue (Bines and Hart, 1982; Bassett, 1975).

1.4.2.2 Growth hormone

Growth hormone is a peptide secreted by the anterior pituitary gland. Growth hormone appears to play a major role in glucose metabolism. Although insulin is the key hormone in glucose metabolism, during ruminant lactation, it is GH that reduces the sensitivity of the peripheral tissues to insulin and thus the uptake of glucose and thereby reduces the rate of glucose clearance from the circulation (McCutcheon and Bauman, 1986; Bauman and McCutcheon, 1986). During GH treatment there are metabolic adaptations in terms of glucose turnover and oxidation to provide the additional glucose required for increased lactose synthesis. The reduction in glucose oxidation during GH treatment provides approximately 30% of the additional glucose required for lactose synthesis (Peel and Bauman, 1987). Bauman *et al.* (1988) reported that exogenous GH increased glucose irreversible loss rate, which was highly correlated with milk lactose secretion in lactating cows and consistent with earlier findings in sheep and goat at peak lactation (Bergman and Hogue, 1967; Annison and Linzell, 1964). Thus, during GH treatment, this increased utilization of glucose for milk synthesis other than oxidation is consistent with earlier findings that GH administration resulted in a reduction in glucose uptake by the hind limb muscles and an increase in glucose uptake by the mammary gland in dairy cows (McDowell *et al.*, 1987). Furthermore Pocius and Herbein (1986) showed that the liver capacity for glucose production from propionate was increased in cows treated with GH. These adaptations ensure that nutrients are effectively partitioned towards the mammary gland for milk synthesis.

Exogenous GH increases milk protein yield (Peel *et al.*, 1981; Bauman and McCutcheon, 1986). In line with this, Hanigan *et al.* (1989) showed that amino acid uptake by the lactating mammary gland increased in response to GH treatment. However, it may be that some of these amino acids were oxidized to provide energy for galactopoiesis rather than being used actively for milk protein synthesis (Metcalf *et al.*, 1994).

The increase in energy demand in early lactation results in a rapid mobilisation of stored lipids. Administration of exogenous GH increases circulating concentrations of NEFA and milk yield (Bauman and McCutcheon, 1986). An increase in NEFA

concentrations during GH treatment is unlikely to reflect a direct effect of GH, but rather it causes alterations in the sensitivity of the adipose tissue to other hormones such as insulin and epinephrine (Peel and Bauman, 1987). During GH administration, the circulating concentrations and irreversible loss rate of NEFA were increased in dairy cows (Bauman *et al.*, 1988) and lactating ewes (Sandles *et al.*, 1988). The irreversible loss rate of NEFA, which increased by 70 – 80% in response to GH, was highly correlated with circulating NEFA and the synthesis of milk fat (Bauman *et al.*, 1988).

The effects of GH appear to be dependent on the energy balance of treated cows. When milk yield responses to GH treatment cause cows to enter a negative energy balance, the concentration of fat in the milk increases. Thus, the response in milk fat yield markedly exceeds the response in milk, with chronically elevated plasma concentrations of NEFA (McCutcheon and Bauman, 1986; Eppard *et al.*, 1985; Peel *et al.*, 1981). Bauman *et al.* (1988) showed that the increase in NEFA irreversible loss rate was highly correlated with the degree of negative energy balance. It has also been observed that during negative energy balance, GH increases the proportion of long-chain fatty acids in milk fat, reflecting an increased mobilisation of body fat reserves (Eppard *et al.*, 1985; Bitman *et al.*, 1984). In contrast, when animals were in positive energy balance, milk fat percentage was not altered by GH treatment, so increases in milk fat yield were similar to increases in milk yield (Peel *et al.*, 1983, 1982). Thus, GH appears to stimulate the synthesis and secretion of all the solids components of milk equally when cows are in positive energy balance while lipid mobilisation and oxidation of NEFA increase when they are in negative energy balance. The increased lipid mobilisation and oxidation of NEFA favours the conservation of limited supplies of other key metabolites such as glucose and amino acids (Peel and Bauman, 1987).

1.4.2.3 Somatostatin

Somatostatin (SS) is a peptide that inhibits GH release; the interplay between SS and growth hormone releasing hormone (GHRH) is believed to govern the typical pulsatile manner of GH secretion (Patel and Srikant, 1985; Tannenbaum *et al.*, 1990). Since SS regulates the release of GH, the secretion of SS is of physiological significance.

In addition to its release from the hypothalamus (Patel and O'Neil, 1988; Plotsky and Vale, 1985; Berelowitz *et al.*, 1981), SS is also secreted from the abomasum, small intestine and pancreas in sheep and stomach of dog (Reddy *et al.*, 1984; Schusdziarra *et al.*, 1978, 1979). SS is a paracrine hormone in the GI tract and pancreas (Taborsky, 1983), and regulates GH secretion by the anterior pituitary gland (Patel and Srikant, 1985; Patel *et al.*, 1982; Tannenbaum *et al.*, 1990; Katakami *et al.*, 1988; Amberdt *et al.*, 1987; Samols and Stagner, 1990; D'Alessio *et al.*, 1989). In addition, Reichlin (1987) has extensively reviewed literature relevant to physiological roles of SS.

If the circulating SS exerts its effect as a true hormone, circulating SS, which passes through the pituitary vascular system, inhibits GH secretion. The finding that preferential binding of SS-28 to SS-28-type receptors in the normal pituitary may indicate that the circulating SS, whether derived from the hypothalamus or other body organs, is capable of exerting an inhibitory effect on pituitary GH secretion (Patel *et al.*, 1990). This extends the findings of Gorewit (1980) who showed that the administration of exogenous SS into the blood decreased plasma levels of GH in dairy cows. Furthermore, exogenous SS abolishes the secretory bursts of GH in dogs (Cowan *et al.*, 1984). Injection of SS into the external pudic artery decreased milk yield in goats, although plasma GH concentrations were not changed (Davis *et al.*, 1996). This, however, suggests that SS has a direct action on the mammary gland in lactating ruminants or at least in goats.

1.4.2.4 Glucagon

Glucagon is a peptide secreted by the α-cells of the pancreas and is a potent hyperglycaemic hormone in ruminants. Glucagon has been shown to be stimulatory to glycogenolysis and, especially, gluconeogenesis in sheep (Brockman and Greer, 1980; Brockman, 1978). Glucagon stimulates the hepatic extraction of gluconeogenic amino acids such as alanine, glutamate, serine, threonine and lactate (Brockman and Bergman, 1975) and thus enhances gluconeogenesis. Propionate is the major glucogenic substrate used in gluconeogenesis in ruminants and its utilization in gluconeogenesis may not be altered by glucagon. Glucagon has little influence on basal glucose production in the lactating cow (Brockman and Laarveld, 1986). However, infusion of glucose into cows early in lactation did not alter plasma glucagon concentrations (Sartin *et al.*, 1985b)

whereas propionate infusion did increase plasma glucagon concentrations (Sartin *et al.*, 1985a). The increased glucagon concentrations in cows early in lactation in response to propionate infusion may be to enhance gluconeogenesis and thereby support an increased demand for glucose for milk synthesis. Together, these results suggest that glucagon may only play a role in glucose metabolism when there is a requirement for blood glucose to be elevated (i.e. during early lactation) or an increased blood concentration of gluconeogenic substrates, for example, increased GI tract absorption of propionate. It may also play a role in promoting a hyperglycaemic state during conditions such as stress (Bines and Hart, 1982).

Unlike the situation in monogastric animals, the involvement of glucagon in protein metabolism is insignificant in lactating ruminants (Brockman and Laarveld, 1986). Although glucagon does not appear to have a direct effect on muscle protein degradation, it indirectly decreases protein synthesis by promoting the uptake of amino acids by the liver for gluconeogenesis and thereby diverting them from muscle protein synthesis (Brockman and Bergman, 1975).

1.4.2.5 Prolactin

Prolactin is a peptide secreted by the anterior pituitary gland. It is structurally related to GH, but exhibits a wider diversity of biological functions compared to GH. Prolactin has been implicated as an important lactogenic hormone. A role for PRL in milk synthesis during established lactation is well established in rats, rabbits, dogs, sows and women (Farmer *et al.*, 1998; Forsyth, 1986), but its effect during established lactation in ruminants is less certain and appears to differ considerably from that in non-ruminants.

A surge in PRL release occurs just before parturition in ruminants and Akers *et al.* (1979) reported that blocking the prepartum release of PRL in dairy cows with bromocryptine, a PRL antagonist, resulted in a 40-50% reduction in subsequent milk production. Lactation may be totally inhibited in non-ruminants by blocking the release of PRL (Farmer *et al.*, 1998; Forsyth, 1986). In contrast, inhibition of circulating PRL to very low levels by PRL antagonists or blocking the milking-induced release of PRL during established lactation has very little effect on milk yield in cows (Akers *et al.*, 1981; Beck *et al.*, 1979; Smith *et al.*, 1974; Karg *et al.*, 1972) or goats (Hart, 1973), although Hooley *et*

al. (1978) observed a 20-30% reduction in milk yield of ewes. Recent findings suggest that milk yield and milk composition are not affected by PRL treatment in dairy cows (Plaut *et al.*, 1987, 1985). Thus, the effects of PRL during established ruminant lactation are in marked contrast to the dramatic decline in milk production observed when PRL secretion is blocked in rats, rabbits, dogs, sows and women (Forsyth, 1986; Farmer *et al.*, 1998), suggesting that PRL plays a crucial role in the initiation of lactation but is apparently without effect during established lactation in dairy cows.

1.4.2.6 Glucocorticoids and catecholamines

Glucocorticoids and catecholamines are secreted by the adrenal cortex and medulla, respectively. Glucocorticoids cause a decrease in peripheral uptake of glucose and increase in gluconeogenesis (Reilly and Black, 1973; Reilly and Ford, 1974). Elevated cortisol levels also reduce protein synthesis and increase catabolism (Reilly and Ford, 1974). Catecholamines provide immediate supplies of glucose and lipid fuels in times of stress and hypoglycaemia (Brockman, 1985), by increasing lipolysis and glycogenolysis (Blum *et al.*, 1982; Thomson *et al.*, 1978). Furthermore, McNamara and Hillers (1986) reported that the immediate postpartum period (i.e. 15 to 30 days) was marked by a decrease in lipogenesis and fatty acid esterification in the bovine adipose tissue. During this period, catecholamine-stimulated release of NEFA and glycerol was increased and the catecholamine-stimulated lipolysis remained elevated for up to 6 months postpartum compared to that of prepartum levels.

1.4.3 Vagal regulation of hormone secretion

Lactation requires both an increased supply of nutrients and development of mechanisms which ensure the preferential utilization of nutrients by the mammary gland. These include development of the GI tract and its associated organs such as liver and dramatic changes in the way in which endocrine factors control nutrient partitioning. In the endocrine control of metabolism during lactation, the strategies adopted by various species of animals, show similarities but there are also some very marked differences. On the one hand many species share the same type of adaptation in which hypoinsulinaemia and diminished responsiveness to insulin in adipose and muscle tissue favour the preferential utilization of nutrients by the mammary gland. On the other hand, during

established lactation, hormones required for the maintenance of lactation differ between species. For example, rabbits sustain lactation in the presence of PRL alone (Cowie, 1969), whereas many of the species of domestic ruminants appear much more dependent on the metabolic effects of GH (Forsyth, 1986). However, in many species, the mechanisms which controls the release of these hormones during lactation are not clearly understood, but the data reviewed in this section suggest that the vagus nerve is involved.

The observations in lactating monogastric animals (Eriksson *et al.*, 1994) as well as in calves and sheep aged 2 to 6 months (Bloom and Edwards, 1985, 1981) on the role of the vagal innervation of the GI tract suggest that the vagal nerve activity could influence the release of various hormones. This has led to the proposal, which is discussed in the following sections that the neural axis, in particular the vagus nerve, could play an important role in the regulation of nutrient homeostasis as well as homeorhesis (Eriksson *et al.*, 1994; Uvnas-Moberg, 1992; Uvnas-Moberg, 1989), and thereby regulate nutrient metabolism in lactating ruminants.

1.4.3.1 Insulin secretion

Although the regulation of insulin secretion has been extensively studied (Sutton *et al.*, 1988; De Jong, 1982; McAtee and Trenkle, 1971; Horino *et al.*, 1968), the mechanisms by which endogenous and exogenous nutrients stimulate insulin release are not well understood in ruminants. It is generally accepted that glucose and other metabolites in the blood plasma have concentration-dependent direct effects on the islets of Langerhans in the pancreas in controlling the rate of insulin secretion. In addition, the existence of an autonomic neural control mechanism for regulating the rate of secretion of the hormone has been strongly suggested by Woods and Porte (1974).

The neural connection between the GI tract, brain and endocrine pancreas is well established in monogastric animals (Ami *et al.*, 1993; Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Kirchgessner and Gershon, 1989; Singer *et al.*, 1989). Many workers have reported that the vagus nerve modulates pancreatic insulin secretion in response to a variety of stimuli. Innervation of the pancreas by a branch of the hepatic nerve from the ventral vagus has been described in rats (Niijima *et al.*, 1990; Yamazaki and Sakaguchi, 1989), such innervation is not clear in ruminants. However, the available

evidence from anatomical and electrophysiological studies suggests that in rats, the vagal branches that innervate the upper GI tract, also innervate the endocrine pancreas (Section 1.2.5.3).

Results from numerous studies indicate that an electrical stimulation of the main trunk of the vagus nerve causes the release of insulin from the pancreatic β-cells in a number of species (Adrian *et al.*, 1983; Bloom and Edwards, 1981; Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Ahren and Taborsky, 1986; Holst *et al.*, 1981; Daniel and Henderson, 1967). Furthermore, pre-treatment with atropine strongly inhibited the rise in plasma insulin in response to electrical stimulation (amplitude = 10 Hz, 5 msec, 13.5 mA, 10 min) of both the ventral and dorsal branches of the thoracic vagi below the heart in dogs (Ahren and Taborsky, 1986). Furthermore, bilateral electrical cervical vagal stimulation-induced insulin secretion was significantly suppressed in rats that underwent gastric or hepatic branch vagotomy (Berthoud and Powley, 1990). Similarly, insulin secretion in response to electrical stimulation of either the left or the right cervical vagi was significantly reduced in rats in which either the gastric or hepatic vagal branches or both were cut (Berthoud *et al.*, 1990). In contrast, insulin release in response to electrical stimulation was not affected in animals that underwent coeliac branch vagotomy. These latter results suggest that vagal control of insulin secretion is not mediated through all branches of the vagus. Further they suggest that, in rats, vagal control is mediated through axons projecting through gastric and hepatic branches of the vagus nerve. Furthermore, anterograde and retrograde tracing methods have demonstrated the occurrence of axonal projections between the GI tract and pancreas (Berthoud *et al.*, 1990; Kirchgessner and Gershon, 1990, 1989 and Section 1.2.5.3). These studies have shown that in the rat, vagal efferent axons run distally from stomach to intestine and selectively terminate on command neurons, which activate intrinsic enteric neurons of the myenteric plexus (Kirchgessner and Gershon, 1989). Many of these enteric neurons enter the pancreas, forming an extensive entero-pancreatic innervation (Kirchgessner and Gershon, 1990; Poulsen *et al.*, 1983). These results are in accordance with the data of Ami *et al.* (1993) who found that vagotomy reduces pancreatic secretion by interrupting vagus-mediated reflexes between the GI tract and the pancreas. Kameyama *et al.* (1993) studied the pancreatic hormone release in response to electrical stimulation of the dorsal vagus nerve of distally

gastrectomized dogs. They found that transection of vagal branches close to the antrum and pylorus interfered with pancreatic hormonal release, indicating the existence of an efferent vagal pathway between the GI tract and pancreas. Furthermore, the use of anterograde and retrograde tracer revealed that the axon projections (preganglionics) innervating the pancreatic β-cells are contained within the DMN of the vagus (Berthoud *et al.*, 1990; Berthoud and Powley, 1990). This is consistent with the findings that stimulation of the DMN of the vagus caused insulin secretion (Laughton and Powley, 1987).

In support of the data from studies in monogastric animals, evidence suggests that electrical stimulation of the vagus nerve causes insulin release in ruminants, although relatively little work has been carried out. Insulin release was significantly increased in response to electrical stimulation of the vagus nerve in sheep and calves (Pierzynowski *et al.*, 1986; Adrian *et al.*, 1983). Pre-treatment of calves with atropine effectively suppressed the rise in plasma insulin concentration in response to vagal stimulation (Adrian *et al.*, 1983; Bloom and Edwards, 1981), suggesting that the vagus nerve modulates insulin release in ruminant species.

Although the neural pathways involved in the nutrient-stimulated insulin secretion are not understood in ruminants, a substantial literature exists for monogastric animals. Rats subjected to hepatic vagotomy showed a significantly reduced insulin secretion following portal glucose injection, when compared with that of controls (Yamazaki and Sakaguchi, 1989). These same workers suggested that the reduction in insulin secretion was due to the interruption of both the afferent and efferent fibres innervating the pancreas through the hepatic branch of the vagus nerve. Cardin *et al.* (1992) found that hepatic vagotomy can disrupt insulin release in response to a portal glucose load. Russek (1963) demonstrated the possible existence of gluco-receptors in the liver, following the observation that i.p. glucose injection caused satiety in dogs. The author suggested that responses from the brain, induced by increased glucose concentrations in the portal blood, which normally occurs after i.p. injection, involve a mechanism closely linked to hepatic gluco-receptors. This mechanism informs the brain about the intracellular concentrations of glucose in hepatocytes. In a study with guinea pigs, Niijima (1982) reported that the hepatic branch of the vagus nerve contains glucose-sensitive afferent nerve fibres and the

mean discharge rate of these fibres is positively related to the concentration of glucose in the portal blood. These conclusions were based on the observations that intra-duodenal infusion of isotonic (5%) glucose solution caused an increase in glucose content in the portal venous blood and this was accompanied by a gradual increase in afferent discharge rate in the hepatic branch of the vagus nerve. Niijima (1982) suggested that in guinea pigs, glucose sensitive afferent nerve fibres in the hepatic branch of the vagus nerve are solely responsible for the induction of insulin secretion by glucose in the portal blood since the frequency of the vagal afferent impulses reaching the hypothalamus through the NTS was dependent upon the glucose concentration in the portal venous blood. Together, these results indicate that there is a vagal signaling pathway between the liver, pancreas and the brain, which is involved in the stimulation of insulin secretion by nutrients in the portal blood of monogastric animals.

While glucose is the main end product of carbohydrate digestion in monogastric animals, propionate is formed in the rumen during microbial fermentation of carbohydrates in ruminants and this is the second most abundant VFA found in both the rumen and blood. The propionate formed in the GI tract is an important stimulus for insulin secretion in ruminants. For example, Bines and Hart (1984) infused mixtures of VFA into the rumen of cattle and found propionate was the major stimulant of insulin secretion. Intra-jugular or intra-femoral or intra-saphenous administration of VFA caused an increased secretion of insulin in cows (Sartin *et al.*, 1985a), goats (De Jong, 1982), sheep (Sano *et al.*, 1995, 1993; Horino *et al.*, 1968; Manns and Boda, 1967) and lambs (Bloom and Edwards, 1985). Increases in insulin concentrations in response to physiological doses of intra-femoral propionate (Sano *et al.*, 1995) or intra-ruminal propionate (Istasse *et al.*, 1987) administration were not accompanied by any change in plasma glucose concentrations. This indicates that the release of insulin is unlikely to be mediated through a direct action of glucose produced from propionate in the liver. Nor is it likely that propionate has any direct physiological significance as a regulator of insulin secretion because most of the propionate absorbed from the GI tract into the portal blood is removed by the liver before reaching the pancreas (Bergman, 1975). Pre-treatment with atropine abolishes insulin secretion induced by i.v. infusion of propionate and butyrate in sheep (Sano *et al.*, 1993) or butyrate in lambs (Bloom and Edwards, 1985), which suggests that the stimulatory

effect of VFA on insulin release is not direct and, therefore, possibly mediated neurally. Glucose is directly absorbed from the GI tract in monogastric animals and the receptors for glucose, which are involved in the vagally mediated portal glucose induced insulin secretion, are present in the area drained by the portal vein (Yamazaki and Sakaguchi, 1989; Niijima, 1986; 1983, 1982). Because propionate is the major substrate for glucose production in ruminants, it is possible that receptors for propionate may also be present in the portal drained viscera. Thus in this model, the afferent signals would originate in the portal area and send signals to the NTS of the vagus nerve on the availability of propionate in the portal blood. The NTS would stimulate the DMN of the vagus nerve, which, in turn, would stimulate insulin secretion through efferent fibres innervating the pancreas. Therefore, it is proposed that the above findings indicate the existence of a vagal neural pathway between the GI tract and its associated portal viscera, brain and pancreas that is important for insulin release during carbohydrate fermentation in the rumen.

Furthermore, information exists for a role of the vagus in the release of insulin in response to exogenous glucose administration in ruminants. Intravenous administration of glucose causes an increased secretion of insulin in sheep (De Jong, 1982) and calves (Bloom and Edwards, 1981). Bloom and Edwards (1981) showed that the increased secretion of insulin following an intravenous infusion of glucose was significantly reduced by treatment with atropine, suggesting the involvement of the vagus nerve in the release of insulin during hyperglycaemia. These results are consistent with findings in monogastric animals that the insulin release in response to intrajugular (Louis-Sylvestre, 1978) or intragastric (Louis-Sylvestre, 1976) administration of glucose was suppressed by vagotomy in rats. Furthermore, the influence of blood glucose concentration on the hypothalamic neurons has been reported in dogs (Anand *et al.*, 1964), suggesting that insulin secretion in response to glucose infusion may involve changes in the activity of neurons in the higher centres.

Several studies have further confirmed that the pancreatic branch, which arises from the hepatic branch of the ventral vagus nerve, is involved in the release of insulin in response to stimuli originating in the oral cavity in monogastric animals. Efferent activity of the pancreatic and hepatic branches of the vagus nerve was increased in response t application of 0.15M Monosodium glutamate (MSG) to the tongue in rats, which las

for about 30 minutes after an application of the solution (Niijima *et al.*, 1990). A similar response to an application of 5% glucose or 10% sucrose solution to the tongue was observed in anaesthetized rats and decerebrated rats, indicating the existence of a reflex centre in the hypothalamus (Niijima, 1991). These responses to oral stimuli suggest that afferent neurons from the oral cavity activate the efferent parasympathetic neurons in the DMN in the hypothalamus, which innervate the pancreatic β-cells and in turn cause insulin release.

1.4.3.2 Somatostatin secretion

Because of its role in the pituitary gland (Skamene and Patel, 1984) and in almost all cell functions in the GI tract (Gyr *et al.*, 1987; D'Alessio *et al.*, 1989; Konturek *et al.*, 1976a; 1976b), any role of vagus in the control of SS secretion would be of great interest.

The release of SS was increased following vagotomy in lactating rats (Eriksson *et al.*, 1994) and vagal stimulation strongly inhibited SS secretion (Holst *et al.*, 1992). In the latter study, the inhibition was mimicked by the stable choline ester carbachol and was abolished by atropine, results which agree with earlier findings (Holst *et al.*, 1983) and, also support the conclusion of an involvement of muscarinic receptors in SS secretion. In *in vitro* studies using an isolated perfused rat pancreas with intact vagal innervation, electrical vagal stimulation inhibited SS release whereas when atropine was also infused SS release was enhanced (Nishi *et al.*, 1987). Further, electrical stimulation of the vagus nerve inhibits the release of SS into the portal blood in cats (Uvnas-Wallensten *et al.*, 1980) and from the stomach and pancreas in the pig (Holst *et al.*, 1981). Although it is generally accepted that the hypothalamic SS inhibits GH secretion from the pituitary gland, it is also possible that circulating SS also affects the secretion of GH since it is produced in large amounts and released into the circulation from the stomach and pancreas in many species of animals including ruminants (Reddy *et al.*, 1984; Schusdziarra *et al.*, 1978). Thus a possible role for the vagus in the regulation of GH secretion through its effect on SS secretion warrants further study because of the strong galactopoietic effect of GH in lactating ruminants.

1.4.3.3 Prolactin secretion

The mechanism of PRL release in ruminants is not well understood. Feeding causes the release of PRL in sows, rats and cows (Samuelsson *et al.*, 1996b; Rojkittikhun *et al.*, 1993; Xie, 1991; McAtee and Trenkle, 1971). However, it has been reported that the vagal nerve activity exerts a tonic stimulatory effect on PRL producing cells in rats (Eriksson *et al.*, 1994). The factors affecting PRL secretion and their vagal regulation are discussed in Section 1.4.5.1.

1.4.4 Cephalic phase insulin release (CPIR)

The sensory perception of food before and during ingestion triggers neural reflexes that result in various motor and secretory responses in the GI tract and its accessory organs. These food-related sensory stimuli such as sight, smell, sound or taste of the food, have always been considered specific and effective elicitors of CPIR, the insulin released preabsorptively in response to these stimuli. Although the functional significance of the cephalic phase reflexes is still unclear, it was proposed that this type of response prepares the viscera for the efficient digestion, absorption and utilization of nutrients (Strubbe and Steffens, 1975).

CPIR is well documented in non-ruminants including humans (Strubbe and Steffens, 1975; Strubbe, 1992; LeBlanc *et al.*, 1991; Berthoud *et al.*, 1980, 1981; Berthoud and Powley, 1990; Berthoud and Jeanrenaud, 1982; Steffens, 1976; Rogers and Blundell, 1989; Yamazaki and Sakaguchi, 1986; Bruce *et al.*, 1987; Teff *et al.*, 1993, 1995; Bellisle and Louis-Sylvestre, 1983) and ruminants (Faverdin, 1986b; Porter and Bassett, 1979; Bassett, 1974a; Lofgren and Warner, 1972; Vasilatos and Wangsness, 1980; Bhattacharya and Alulu, 1975; Chase *et al.*, 1977). The results from these studies have clearly indicated that the food-related CPIR responses were not preceded by any significant changes in blood glucose concentrations. For example, Strubbe and Steffens (1975) showed that in rats, ingestion of a meal caused an immediate release of insulin in the absence of any significant changes in glycaemia. Lambs at 4 days of age and older as well as infant rabbits aged 1 to 15 days, secreted insulin within 5 minutes of suckling without any change in plasma glucose concentrations (Porter and Bassett, 1979). Faverdin

(1986b) demonstrated that cows released insulin in response to the visual presentation of feed during a prefeeding period of 5 minutes.

It was suggested that the cephalic phase reflexes including CPIR are a group of physiological responses that occur after activation of the vagus nerve through food-related sensory stimulation (Strubbe and Steffens, 1975; Berthoud *et al.*, 1981; Taylor and Feldman, 1982). The findings that meal associated CPIR was almost completely blocked by prior i.v. administration of atropine in rats (Berthoud and Jeanrenaud, 1982; Strubbe, 1992) and lambs (Porter and Bassett, 1979) and by gastric vagotomy in rats (Berthoud and Powley, 1990; Louis-Sylvestre, 1976) suggest that CPIR response is mediated through the vagus nerve. Furthermore, sensory stimuli such as the application of solutions of glucose, sucrose or MSG that originate in the oral cavity cause an increase in efferent discharge rate of the pancreatic branch of the vagus nerve in rats (see Figure 1.3) (Niijima, 1991; Niijima *et al.*, 1990; Grill *et al.*,1984; Louis-Sylvestre, 1976). Niijima *et al.* (1990) observed that CPIR, which occurred 3 minutes after application of 2M glucose or 0.15M MSG to the oral cavity in unanaesthetized rats, was paralleled by an increased efferent activity of the pancreatic nerve. These results suggest that CPIR response elicited immediately after ingestion of food is mediated through the vagus nerves in monogastric and ruminant animals. However, further investigations are necessary to confirm the vagal pathways involved in CPIR in ruminants.

1.4.5 Suckling and the regulation of nutrient metabolism

An increased plane of nutrition is a prerequisite for maximizing milk yield during lactation, particularly in the dairy cow, which has been bred for milk production. During lactation a higher percentage of nutrients is diverted towards the mammary gland (Section 1.3), because lactation is a physiological process where mothers use post-absorptive nutrients and body reserves extensively. Thus, in order to increase the efficiency of these processes during lactation, various types of integrated mechanisms begin to operate during this period. For example, suckling or milking in dairy breeds, influences the release of several hormones from the pituitary gland and the GI tract (Samuelsson *et al.*, 1996a, 1996b; Svennersten *et al.*, 1989; Eriksson *et al.*, 1994). Thus there are potential relationships between suckling/milking and the release of these hormones that are

necessary for the partitioning of nutrients between the mammary gland and extramammary tissues and also to adjust development of the GI tract. These relationships, together with the involvement of the vagus nerve, are discussed in this section.

1.4.5.1 Suckling and pituitary hormones

Initiation of lactogenesis and the maintenance of established lactation are under the influence of pituitary hormones. However, there are species differences in the maintenance of an established lactation. For example, lactation in many monogastric animals depends upon PRL whereas normal concentrations of PRL do not limit milk secretion during established lactation in ruminants (Section 1.4.2.5). In contrast, GH is galactopoietic during established lactation in ruminants and its removal inhibits lactation in goats (Cowie *et al.*, 1964). While these two pituitary hormones are important in the maintenance of mammary secretory cell activity, OT is essential for milk ejection.

Oxytocin is synthesized in neurons of the SON and PVN in the hypothalamus. This neurosecretory product is transported down the axons in vesicles complexed with neurophysin and stored in axon terminals in the posterior (neural lobe) pituitary gland. The role of OT in milk removal was first demonstrated by Andersson (1951) following the pioneering study by Ely and Peterson (1941) who showed that OT is involved in the control of milk ejection. Plasma OT levels rise in response to each suckling episode.

It is well recognised that during lactation milk production is reduced if nutrient intake falls. In addition to a direct effect of nutrient intake on the availability of nutrients to the mammary gland, it has also been proposed that milk production is reduced by a reduced flow of afferent vagal impulses from the GI tract to the brain (Samuelsson *et al.*, 1996b; Uvnas-Moberg, 1989). In support of this, Eriksson *et al.* (1994) observed that suckling-associated OT release was significantly decreased in vagotomized rats compared with that of controls. This reduction in OT secretion was associated with a reduction in milk yield. Svennersten *et al.* (1995) demonstrated that feeding dairy cows during milking enhanced milking-induced OT release and milk production, whereas cows deprived of food had low basal and milking-induced OT levels. These results agree with the findings of Samuelsson *et al.* (1996b) who have shown that milking-induced OT secretion tended to be lower after only 20 h of feed deprivation and was significantly reduced 44 h after the

last feeding period. These results are in line with observations that feeding causes OT release in dogs, sows and rats (Uvnas-Moberg *et al.*, 1985; Verbalis *et al.*, 1986). Together, these findings with the results of others (Stock and Uvnas-Moberg, 1988) suggest that impulses from the GI tract are of importance for OT secretion and so a reduction in OT release resulting in decreased efficiency of milk ejection could be a factor in the reduced milk production of vagotomized rats (Eriksson *et al.*, 1994). Furthermore the effect of food deprivation and refeeding on OT release is probably mediated through the vagus nerve responding to the amount of food in the stomach.

Indeed, the influence of vagal nerve activity on plasma levels of OT is well documented. Afferent electrical stimulation of the subdiaphragmatic branches of the vagal nerves immediately elevated plasma OT concentrations (Stock and Uvnas-Moberg, 1988). In addition, systemic and central application of CCK stimulates OT secretion (Neumann *et al.*, 1994), which is a vagally-mediated phenomenon (Smith *et al.*, 1985) (see also Section 1.4.1). Thus the release of OT in response to i.p. injection of CCK and the prevention of this effect by vagotomy also suggests that the vagus nerve influences OT secretion (Verbalis *et al.*, 1986; Renaud *et al.*, 1987). The involvement of the autonomic nervous system is well supported by the study of Svennersten *et al.* (1992) who have shown that the anticholinergic agent, atropine, inhibited the release of OT, suggesting that a mechanism, probably centrally acting, and involving muscarinic type receptors, may be responsible for the release of OT.

Anatomical and immunohistochemical evidence is accumulating to support the concept that the OT producing neurons are influenced by the vagal neurons and *vice versa* (McCann and Rogers, 1990). There are potential interrelationships between two vagal nuclei, the DMN and NTS and two OT producing nuclei, PVN and SON (Figure 1.3). In the rat, an injection of true blue into the DMN of the vagus labelled a tract of OT-containing neurons in the PVN (Sawchenko and Swanson, 1982). In support of this, Siaud *et al.* (1989) found that OT-immunoreactive fibres are present in both the NTS and DMN of the vagus in rats and guinea pigs. Later Sawchenko *et al.* (1988) demonstrated an anatomical basis for the mechanism responsible for changes in OT release and its relationship to the vagal nuclei in the hypothalamus. They identified the prominent projections of beta-inhibin containing fibres from the NTS to oxytocinergic neurons in the

PVN. Furthermore, the findings that an injection of beta-inhibin into the PVN caused the release of OT, suggest that sensory information received by the NTS are conveyed to OT-producing neurons in the PVN and beta-inhibin is most likely to be the messenger in this neural pathway. A neural communication between the NTS and OT producing neurons was also demonstrated by Van der Kooy *et al.* (1984) while a direct neural communication was described between the PVN and DMN (Saper *et al.*, 1976; Sims and Lorden, 1986). Sims and Lorden (1986) demonstrated that rats developed hyperinsulinaemia and obesity if lesions were produced in the PVN and these effects were reversed by vagotomy. Further evidence to support the interconnection between the PVN and DMN of the vagus was provided by Rogers and Hermann (1986). They reported that in rats, microstimulation of the PVN neurons or microinjection of OT into the DMN of the vagus elicited significant increases in gastric acid secretion and bradycardia, two functions regulated by the DMN of the vagus nerve. This confirmed the previous findings that cervical vagal stimulation-evoked gastric acid secretion was suppressed by lesions produced in the PVN (Rogers and Hermann, 1985). Moreover, Rogers and Hermann (1986) reported that microinjection of an OT antagonist, dET2Tyr(Et)Orn8 Vasotocin (ETOV), into the DMN blocked these effects on gastric acid secretion and heart rate whereas injection of an equal volume of artificial cerebrospinal fluid vehicle solution into this region of the DMN had no effect on either the changes in acid secretion or cardiac activity. All these results suggest that the NTS and DMN of the vagus, and PVN are closely interconnected neurally, comprising a common pathway(s) for the exchange of information between these nuclei (see Figure 1.3). Verbalis *et al.* (1986) and Sawchenko *et al.* (1988) suggested that feeding-induced OT secretion might be a consequence of the release of a GI tract hormone, such as CCK, which activates the oxytocinergic neurons through the NTS in the hypothalamus.

Prolactin is released as a result of milking in dairy cows (Samuelsson *et al.*, 1996a; Samuelsson *et al.*, 1996b; Koprowski and Tucker, 1973; Johke, 1969), goats (Hart and Linzell, 1977; Hart, 1975, 1973) and sheep (Fell *et al.*, 1972b) or suckling in dairy cows (Bar-Peled *et al.*, 1995), rats (Eriksson *et al.*, 1994) and women (Tay *et al.*, 1996; Yokoyama *et al.*, 1994). The suckling-induced release of PRL was reduced in lactating vagotomized rats (Eriksson *et al.*, 1994), which is a probable reason for their decreased milk yields, as measured by the weights of their litters. Feed deprivation has been shown

to reduce the basal levels of PRL in lactating sows (Rojkittikhun *et al.*, 1993), rats (Xie, 1991) and cows (Samuelsson *et al.*, 1996b; McAtee and Trenkle, 1971), but can be restored within 10 min of refeeding (Rojkittikhun *et al.*, 1993). The significant reduction in suckling-related PRL secretion in vagotomized rats suggests that the vagal nerve activity may exert a tonic stimulatory effect on PRL producing cells (Eriksson *et al.*, 1994) through a mechanism involving NTS and PVN of the hypothalamus. The same type of mechanism is probably operating in lactating goats. Hart and Linzell (1977) compared the effect of milking denervated transplanted (to the neck) mammary glands with that of intact mammary glands in goats and found that the concentration of PRL in the circulation rose after milking the intact glands but not the transplanted glands. They also observed that milking only one teat produced less PRL secretion than after milking both teats for the same time. These findings suggest the involvement of a neuroendocrine mechanism in the milking-associated release of PRL in goats. Thus, it is probable that the mammary sensory signals emanating from tactile stimulation of the teats stimulate the secretion of hypothalamic neurotransmitters such as dopamine and serotonin, which, in turn, influence the release of hypothalamic PRL releasing factor(s) (vasoactive intestinal polypeptide (VIP)) or PRL inhibiting factor, which is probably dopamine, into the hypophyseal portal veins (see Figure 1.3). For example, serotonin appears to stimulate the release of VIP while inhibiting dopamine, because dopamine itself is considered to be PRL inhibiting factor (Brown, 1994; Rolandi *et al.*, 1987; Cowie and Tindal, 1975). Thus it may be assumed that the hypothalamic neurons that produce these PRL releasing neural factors are potentially interrelated with that of the vagal nuclei.

Taken together, all these results suggest that the vagal innervation of the GI tract is of importance in suckling-induced and feeding-induced OT and PRL secretion and thus milk production of the rats. It is possible that at the hypothalamic level, neural signals originating from the mammary gland as a result of suckling or milking in dairy breeds (Eriksson *et al.*, 1994; Tindal, 1978; Tindal and Knaggs, 1975) or the GI tract as a result of food arrival (Svennersten *et al.*, 1995, 1992, 1990; Samuelsson *et al.*, 1996b) are conveyed to OT and PRL releasing factor(s)-producing neurons through the vagal nuclei, the NTS and DMN. However, the exact nature of this neuroendocrine axis has not been investigated in a ruminant.

1.4.5.2 Suckling and GI tract hormones

During lactation, the increased demand for nutrients is met mainly by increases in voluntary food intake. This increased intake leads to a development of the GI tract that increases its capacity to process extra food. This includes increases in the size of the various organs, digestive ability and the absorptive capacity of the GI tract (Section 1.3.3). It was proposed by Uvnas-Moberg and colleagues that the release of GI tract hormones during feeding (McLeay and Titchen, 1975; McLeay and Bell, 1980) and suckling (Uvnas-Moberg *et al.*, 1984; Eriksson and Uvnas-Moberg, 1990; Linden *et al.*, 1987; Uvnas-Moberg and Eriksson, 1983) is important for changes in the GI tract associated with lactation.

There are a number of polypeptide hormones released by the GI tract both into the circulation and into the GI tract lumen. Of these, CCK is one that has been given much attention in lactating monogastric animals (Linden *et al.*, 1990, 1987 and Section 1.4.1). A close relationship between activation of the vagal nerves and CCK secretion is well established (Shillabeer and Davison, 1985 ; Mercer *et al.*, 1992 ; Farningham *et al.*, 1993). Linden *et al.* (1990) reported that the increased levels of CCK in response to suckling were abolished by vagotomy in lactating rats, suggesting that the release of CCK is vagally mediated. Furthermore, lesions of the midbrain which disrupted the milk-ejection reflex, also blocked the suckling induced rise in CCK levels in lactating rats, suggesting the existence of a neuro-endocrine axis between the GI tract, brain and the lactating mammary gland (Linden *et al.*, 1990). Suckling-induced CCK release, in addition to its effects on food intake, appears to be important during lactation since CCK stimulates the release of OT via the vagus nerve (Verbalis *et al.*, 1986; Renaud *et al.*, 1987; Sawchenko *et al.*, 1988; Neumann *et al.*, 1994 and Section 1.4.5.1).

The release of insulin during suckling has been observed in lactating dogs (Eriksson *et al.*, 1987; Uvnas-Moberg and Eriksson, 1983), sows (Uvnas-Moberg *et al.*, 1984), rats (Eriksson *et al.*, 1994) and women (Franceschini *et al.*, 1990; Widstrom *et al.*, 1984). The mechanism by which suckling stimulates the release of insulin is not understood. Since an electrically activated vagal nerve leads to a stimulation of insulin secretion (Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Adrian *et al.*, 1983; Holst

et al., 1981; Daniel and Henderson, 1967), it is possible that suckling-stimulated release of insulin is a consequence of activated vagal nerves. Thus, sucking the teats may cause a reflex activation of the hypothalamic vagal nuclei such as the DMN of the vagus, which in turn through vagal efferents, stimulate insulin release from the pancreas. Eriksson *et al.*(1994) have observed that suckling-related release of insulin in the vagotomized rats was decreased but it did not achieve a statistically significant level. The authors suggested that a period of high blood glucose concentrations might have preceded suckling in the vagotomized rats and thus partially counteracting the direct effect of the vagotomy on insulin concentration.

Suckling causes the release of glucagon in lactating dogs (Eriksson *et al.*, 1987), and sows (Algers *et al.*, 1991). Although the mechanism underlying this secretion is not known, this may be a vagally dependent mechanism since activation of the vagal nerves is known to cause glucagon release (Uvnas-Moberg, 1983; Holst *et al.*, 1981). However, Eriksson *et al.* (1994) reported that glucagon concentrations were not affected by suckling in control or vagotomized rats. It appears that further investigations are necessary to elucidate the role, if any that the vagus has in the control of glucagon secretion.

The vagal nerve activity on SS secretion is inhibitory (Holst *et al.*, 1992, 1981; Nishi *et al.*, 1987; Uvnas-Moberg, 1987; Uvnas-Wallensten *et al.*, 1980) and discussed in Section 1.4.3.2. The normal rise in SS in response to suckling was enhanced following vagotomy in lactating rats (Eriksson *et al.*, 1994). This may be due to the elimination of inhibition following vagotomy, or as a consequence of increased sympathetic activity (Short *et al.*, 1985) or both. In contrast, Samuelsson *et al.* (1996b) did not observe an increase in SS secretion in response to milking in food deprived cows. Rather, SS secretion tended to decrease during milking. However, milking with simultaneous feeding causes high plasma concentrations of SS (Samuelsson *et al.*, 1996a). This suggests that the decreased concentrations of SS during milking in food deprived cows is likely to be due to the fasting since feeding is shown to be a strong stimulus for SS secretion in cows (Svennersten *et al.*, 1989) and dogs (Schusdziarra *et al.*, 1978, 1979). However, because SS secretion is stimulated by sympathetic nervous system through beta-adrenergic mechanism (Short *et al.*, 1985), it is still possible that the vagal activity (parasympathetic) inhibits SS secretion since these nervous systems are known to act in an opposite manner.

Gastrin produced in the gastric antrum (in the abomasum of ruminants) is chemically related to CCK (the five C-terminal amino acids are identical). The release of gastrin is stimulated by the presence of food in the abomasum or the intestine (Reynolds *et al.*, 1991). Gastrin stimulates gastric juice secretion although in ruminants secretion is continuous (McLeay and Titchen, 1975).

Suckling causes the release of gastrin in lactating sows (Uvnas-Moberg *et al.*, 1984), dogs (Linden *et al.*, 1987) and cows (Svennersten *et al.*, 1989), but machine milking in fed lactating cows did not increase the release of gastrin (Samuelsson *et al.*, 1996a). This was also the case in another study with sows in which suckling did not influence plasma gastrin (Algers *et al.*, 1991). However Algers *et al.* (1991) fed their sows 2-4 h before the experiments were performed whereas a longer fasting period was used by Uvnas-Moberg *et al.* (1984) so there may have an undetected increase in gastrin concentration in response to suckling concealed by increased baseline concentrations. This was further supported by the findings of Samuelsson *et al.* (1996b) who observed a significant increase in gastrin in response to machine milking in fasted cows. It is likely that not only the presence of food influences gastrin secretion but also a neural factor, possibly vagal, may be involved in stimulating gastrin secretion. For example, Bloom *et al.* (1978) reported that administration of atropine strongly suppressed the initial rapid rise in plasma gastrin concentration during the first 10 minutes of feeding in calves.

Taken together, all these results suggest that the role of the suckling-related release of GI tract hormones is not only to help to integrate GI tract functions, but also to regulate the postprandial metabolism of ingested food (Eriksson *et al.*, 1994; Schusdziarra and Schmid, 1986; Schusdziarra *et al.*, 1979). The major changes taking place in the development of the GI tract during lactation in ruminants, described earlier in this review, may be directly associated with increased food intake but the changes in hormone concentrations associated with suckling or milking may also play an important part. These changes ensure that the increased demand for nutrients created by the active mammary gland is met by enhanced digestion and metabolic processes. It would seem that such a system would require the integration of vagal afferent and efferent activity within the brain. Although it is unknown whether such a neuroendocrine axis exists in lactating ruminants, Andersson *et al.* (1958) demonstrated that in response to suckling, lactating

goats started ruminating, a vagally-mediated phenomenon, suggesting that there is a neural relationship between suckling and the GI tract, apparently mediated through the activation of the vagal nerve. Although a majority of the responses during suckling described above were from studies of monogastric animals, it is also possible that the same responses occur in ruminants.

1.5 SUMMARY

During lactation, milk synthesis is directly affected by food intake. Increased demand for nutrients created by the mammary gland is met primarily by nutrient absorption from the GI tract. Control of intake is a complex process in which metabolites, blood-borne hormones, and neural mechanisms appear to play different roles. However, the neural mechanisms originating in the GI tract may be necessary in monogastric animals because secretion of most of GI tract and pituitary hormones is, at least in part, under direct influence of the vagus nerve. In dairy breeds, optimum milk production is also achieved by a combination of maximized effects of food intake, GI tract adaptations, absorption, and postabsorptive nutrient metabolism. When no food reaches the GI tract milk synthesis and milk ejection is blocked in cows (Samuelsson *et al.*, 1996b; Samuelsson *et al.*, 1993, Uvnas-Moberg, 1989). In ruminants, the plasma levels of the two pituitary hormones, PRL for establishment of lactation and GH for the maintenance of established lactation, are influenced by feeding (Bines *et al.*, 1982; Tindal *et al.*, 1982; McAtee and Trenkle, 1971). The fact that vagotomized rats do not produce enough milk, despite eating normally, suggest that the presence of food in the stomach and intestine facilitates the release of hormones from the pituitary, particularly PRL, OT and perhaps GH, through activation of vagal impulses originating in the GI tract (Eriksson *et al.*, 1994). This indicates that the presence or absence of nutrients in the GI tract influences milk synthesis/removal in the mammary gland via vagally activated mechanism(s). When lactating cows are underfed or deprived of feed they produce less milk due to a lack of nutrients. However, the exchange of information between the GI tract and mammary gland on the nutritional status may be influenced by the vagal innervation of the GI tract (Uvnas-Moberg, 1992, 1989). In addition, suckling in monogastric animals appears to be an important stimulus for milk synthesis. It affects milk synthesis by affecting secretion of

various hormones, most of which are known to be moderated by the vagus nerve. However, it is not known whether changes in the concentrations of blood hormones associated with milking in dairy cow play a significant role in milk synthesis. Therefore, it appears that the vagal pathways between the GI tract and brain and the mammary gland are important in the process of milk synthesis in monogastric animals, but the physiological role of this neuroendocrine axis is largely unknown in ruminant animals.

1.6 PURPOSE AND SCOPE OF THE INVESTIGATION

The role of the vagal innervation of the GI tract in the control of food intake, release of GI tract and pituitary hormones and milk production have been extensively studied in monogastric animals. Although many authors have investigated the nutritional and endocrinological mechanisms regulating milk synthesis and secretion in ruminants, the neuro-endocrine axis between the GI tract and brain and mammary gland has received much less attention. This is especially so for the neural mechanisms underlying insulin secretion in lactating ruminants.

The objectives of this study were to:

1. Examine the effects of cutting the vagal innervation to the GI tract (selective vagotomy) of the sheep on changes in food intake and the efficiency of digestion;

2. Examine the effects of selective vagotomy on the release of insulin in response to intra-jugular administration of glucose and explore the mechanism by which observed effects might occur, and;

3. Examine the effects of selective vagotomy on milk production by the mammary gland of the ewe and explore the amplitude and pattern of hormone release that might be associated with the observed effects.

A deeper understanding of the neural relationships between the GI tract, brain, pancreas, and mammary gland may have important practical consequences in the management of domestic ruminants during lactation.

CHAPTER 2

THE VAGAL CONTROL OF INSULIN RELEASE AND MILK YIELD IN LACTATING EWES

2.1 ABSTRACT

The role of the vagal innervation of the abomasum and upper small intestine in the release of insulin and regulation of milk yield was studied in lactating ewes. Vagal fibres innervating the abomasum, pylorus and duodenum were sectioned in 3 lactating ewes (ADV); 3 control lactating ewes underwent sham-operations (SO). Ewes were milked twice daily throughout the experiment and the milk volumes and fat content measured. Residual milk was measured twice. Basal and milking-associated changes in GH, PRL and NEFA concentrations in plasma were determined. Digestibility of DM and N was also determined. Seven and 14 days after the surgery, all animals were given an intravenous injection of glucose (100 mg/kg bw) and jugular blood samples collected for glucose and insulin determinations. Differences in DM and N digestibilities between ADV and SO groups were not significant. Milk yield was significantly ($P < 0.05$) lower in the ADV group than in the SO group only on day one after surgery while fat yield was reduced ($P < 0.05$) for the first two days after surgery. Basal concentrations of PRL and GH in the plasma and their release in response to milking were not affected by vagotomy. Basal concentrations of NEFA were not different between treatment groups. Following the glucose challenges, the plasma concentration of glucose declined more slowly in the ADV group than in the SO group ($P < 0.05$). This resulted in a larger, baseline corrected, total area under the glucose response curve for the ADV group ($P < 0.01$) compared to the SO group at both day 7 and 14. Glucose injection caused a significant ($P < 0.05$) increase in insulin release in the SO ewes, but not in the ADV ewes, at both days 7 and 14. Baseline corrected area under the insulin response curves after glucose injection in the ADV group was significantly ($P < 0.01$) lower than that for the SO group at both days 7 and 14. These results indicate that vagal innervation of the stomach (abomasum), and duodenum modifies the pancreatic release of insulin in response to hyperglycaemia induced by intravenous glucose. A reduction in milk yield on day one after surgery in the ADV ewes probably reflected less efficient milk ejection, a reflex event associated with circulating concentration of plasma OT. This hypothesis was supported by the finding that the ADV animals had depressed overall fat concentration in the milk. In contrast, residual milk volume did not differ between the ADV and SO groups, indirectly suggesting that

plasma OT concentrations were not affected by vagotomy. However, further investigation is needed to confirm this effect on milk yield.

2.2 INTRODUCTION

The demand for nutrients is greatly influenced by the physiological status of the animal. Lactation is the phase of the reproductive cycle during which the demand for nutrients is particularly high to support milk synthesis by the mammary gland. A substantial proportion of the nutrients absorbed from the GI tract or released from body reserves are diverted to the active mammary gland for milk synthesis, particularly in domesticated ruminants bred for dairying production. A preferential partitioning of these nutrients towards the mammary gland during lactation is regulated by homeorhetic mechanisms (Bauman and Elliot, 1983; Bauman and Currie, 1980), which are more complex than simple substrate supply to the mammary gland. However, mechanisms regulating nutrient partitioning and milk synthesis are not fully understood in ruminants.

During lactation, the increased demand for nutrients created by the active mammary gland is met primarily by an increased nutrient absorption from the GI tract. Nevertheless, milk synthesis is not increased to the same extent when nutrients are infused directly into the systemic circulation, bypassing the GI tract. It was argued in Chapter 1 that systemic infusion of nutrients such as glucose and amino acids did not increase milk synthesis in goats and cows (Griinari *et al.*, 1997a, 1997b; McGuire *et al.*, 1995; Neville *et al.*, 1993; Tesseraud *et al.*, 1992; Metcalf *et al.*, 1991; Laarveld *et al.*, 1981; Hove, 1978a; Linzell, 1967). In contrast, it was also argued that abomasal infusion of casein combined with the insulin clamp increased milk and protein yields and tended to increase milk fat yield (Griinari *et al.*, 1997a, 1997b). Milk protein yield was 10% greater than baseline values when infused casein alone, but increased by 28% when combined with insulin clamp (Griinari *et al.*, 1997a). This suggests that systemic infusion of nutrients do not change milk yield but nutrients absorbed through the GI tract are important in determining milk composition and the amount of milk produced by the mammary gland (Guinard *et al.*, 1994; Hurtaud *et al.*,1993; Rogers *et al.*, 1984; Clark *et al.*, 1977; Spires *et al.*, 1975). On the other hand, results obtained by Griinari *et al.* (1997a) indicate that either insulin stimulates milk synthesis during a period of an increased availability of circulating

nutrients, or the presence of nutrients in the GI tract triggers some as yet unknown mechanisms which favour the insulin action on the ruminant mammary gland. However, the failure to stimulate milk synthesis in response to systemic administration of nutrients does not support the former argument. Therefore, the availability of substrates to the mammary gland is probably not rate limiting for mammary metabolism and milk synthesis and thus, the response of the mammary gland to nutritional status may involve the recognition of nutrient input by the GI tract and signalling of this input to the mammary gland (Section 1.4.2.1).

It has been proposed that during lactation milk production is reduced as nutrient intake falls due to a reduced flow of afferent vagal impulses from the GI tract to the brain (Uvnas-Moberg, 1989; Samuelsson *et al.*, 1996b). In support of this, severance of the vagal innervation to the GI tract of the lactating rat reduces milk yield (Eriksson *et al.*, 1994), which is associated with a reduction in suckling-associated concentrations of blood hormones such as PRL, OT and insulin in the circulation. Changes in the release of these, and other (SS and VIP) hormones due to the interruption of the vagal innervation of the GI tract could be instrumental in reducing milk yield. These observations suggest that although milk yield is directly moderated by feed intake, a neural mechanism, possibly the vagus nerve, may be involved in signalling the nutrient status to the mammary gland in ruminants.

Glucose is an essential metabolite in all mammalian species and its concentration in the blood is maintained within a relatively narrow range by a variety of physiological mechanisms. The regulation of glucose metabolism in the lactating ruminants is very important since the mammary gland has a large demand for glucose for the synthesis and secretion of milk solids, in particular, lactose. In this context, insulin plays a central role and its release from the pancreas in response to elevated glucose concentrations are well known. Although insulin does not have any direct effect on the ruminant mammary gland, it regulates the supply of substrates to the mammary gland by altering peripheral extramammary metabolism (Tesseraud *et al.*, 1992; Metcalf *et al.*, 1991 and Section 1.4.2.1). It is also well known that vagal reflexes affect the functioning of the GI tract and its accessory organs, including the pancreas and Woods and Porte (1974) suggested that the sensitivity of the pancreas to glucose is influenced by the autonomic nervous system.

Consequently, many studies have focused on the role of the vagus nerve in regulating insulin secretion during hyperglycaemia in a variety of species (Singer *et. al.*, 1989; Niijima, 1986; Bloom and Edwards, 1982; Daniel and Henderson, 1975), but not in mature ruminants. Therefore, the influence of the autonomic nervous system on the partitioning of the nutrients between the mammary gland and other body tissues and on insulin release in a lactating ruminant is of special interest.

The experiments described in this Chapter were undertaken to study the effects of cutting the vagal innervation of the abomasum, pylorus and duodenum on milk yields, digestibility of DM and N, and energy balance. This study also investigated pancreatic insulin secretion in response to intra-jugular glucose administration.

2.3 MATERIALS AND METHODS

2.3.1 Animals, housing and acclimatization

Six Romney ewes at 3-4 weeks of lactation (mean [±SEM] bw 48.5±3 kg) were obtained from Massey University farms, Palmerston North. They were housed individually in metabolism crates indoors in a temperature controlled room (17-18 °C) and given water *ad libitum* at all times. All animals were fed lucerne pellets and lucerne chaff *ad libitum* over a one week acclimatization period, during which time they were accustomed to housing, handling, feeding and other routine maintenance procedures. All procedures involving lactating sheep were approved by the Massey University Animal Ethics Committee.

2.3.2 Surgical procedures

2.3.2.1 Vagotomy of the abomasum, pylorus and duodenum

Surgery to vagotomise the abomasum and duodenum was carried out using full aseptic procedures and under general anaesthesia. Food, but not water, was withheld for 12-14 h before surgery. Anaesthesia was induced by Saffan (Pitman-Moore New Zealand Ltd., 33 Whakatiki Street, Upper Hutt, New Zealand) injected into a jugular vein at a dose rate of 2.5 ml per 5 kg bw. The sheep were then intubated and anaesthesia maintained by inhalation of a mixture of oxygen and Halothane (ZENECA Ltd., Macclesfield, Cheshire,

UK) through the endotracheal tube. Each sheep was placed in left lateral recumbency and the right flank clipped and cleaned thoroughly with Hibitane (ICI Pharmaceuticals, ICI Australia Operations Pty Ltd., 1 Nicholson Street, Melbourne Victoria) in 70% alcohol. A right paracostal incision was made 14-16 cm long and 2-4 cm caudal to the last rib to expose the abomasum, duodenum and lesser omentum.

The abomasal, pyloric, and duodenal branches of the ventral vagus nerve (see No. 1, 2, & 3 of Figure 1.1) were identified in the lesser omentum. Sections 0.5-2.0 cm long were removed from each nerve and fixed in Bouin's solution for later confirmation of the presence of neural tissue. Vagal branches supplying the rumen, reticulum, and omasum were left intact in all sheep. Special care was taken during removal of the nerves to avoid damage to the accompanying blood vessels. The control animals underwent sham-operations on the same days as the vagotomized sheep, during which all procedures except the severing of the nerves were performed.

After completion of the vagotomy, the laparotomy incision was closed by individually suturing the muscle layers with 2-0 Dexon II (Davis & Geek, Cyanamid of Great Britain Ltd, Gosport, Hampshire, U.K.), and the cut edges of the skin closed with Michelle (AESCULAP, Germany) clips.

Each animal received 5 ml of Streptopen (Pitman-Moore New Zealand Ltd., 33 Whakatiki Street, Upper Hutt, New Zealand) i.m. and 1 ml Temgesic, (Reckitt and Colman Products, U.K.) s.c. at surgery. Following full recovery from the anaesthetic, animals were returned to their metabolism crates. Further antibiotic treatment (5ml Streptopen once per day, i.m.) was given and rectal temperature was recorded for the first two days after surgery.

2.3.2.2 Catheterisation of the jugular veins

Catheters (polyethylene tube: ID - 0.8 mm; OD - 1.2 mm) for blood sampling or the intravenous infusion of glucose solution were inserted into each jugular vein at least 2 days before the start of an experiment. All catheterizations were carried out under aseptic conditions and after local anaesthetic (0.5-1.0 ml of 2% lignocaine) had been injected under the skin overlying the vein. The catheters were filled with sterile heparinized saline

(100 i.u./ml of 0.9% NaCl) and inserted into the jugular vein for 8-10 cm. The external end of each catheter was fitted with a blunted 19 gauge needle, capped and taped to the neck. Each catheter was flushed and refilled with heparinized saline every morning.

2.3.3 Experimental design

2.3.3.1 Feeding

All animals underwent surgery and were randomly allocated to either abomasal and duodenal vagotomized (ADV; n=3) or sham-operated (SO; n=3) groups. After surgery each SO ewe was paired with a ADV ewe and fed the *ad libitum* intake of the paired ADV ewe. All animals received their daily meal of 50:50 lucerne chaff and lucerne pellets (12 MJ ME/kg DM) once a day at 0700 h. The amount of food ingested by each ADV ewe was measured on the following morning (24 h intake) by weighing the refusals. The amount of food ingested was calculated and offered to the SO ewe of each pair so that intake of SO ewes was always 24 h behind that of the ADV ewes. This lag was taken into account when statistical analyses were done. Samples of lucerne chaff and lucerne pellets were stored frozen at different time to measure DM and N contents.

2.3.3.2 Milking

All animals were milked using a milking machine twice daily at 0900 and 1500 h and milk volumes and milk fat percentage were recorded. On post-operative (PO) days 8, 9 (period 1), 16 and 17 (period 2), ewes were milked normally and volumes recorded and then after milking they were injected with 0.1 ml (1 iu) OT into a jugular vein, milked out again and volumes recorded. Fat content of these samples were measured using a mini centrifuge.

2.3.3.3 Milking-associated release of hormones and metabolites

Pre- and post-milking associated release of GH, PRL and NEFA was studied on post-operative days 6 and 13. Blood samples were taken at -30, -20, -10, 5, 15, and 30 min in relation to the afternoon milking on PO days 6 and 14.

2.3.3.4 Digestibility and nitrogen balance

Urine and faeces were collected daily for 4 days on PO days 3-6 (BL-1) and on days 11-14 (BL-2). Urine was collected into plastic buckets containing 200 ml of 2% HCl to prevent volatilisation of NH_3. Urine volume and weight of faeces were recorded and 10% sub-samples stored frozen and pooled across 4 days for each animal for the analyses of DM and N.

2.3.3.5 Glucose challenge

On PO days 7 and 14 all ewes were given a glucose challenge consisting of a bolus injection of glucose (100 mg/kg bw) through one jugular catheter inserted 2-3 days previously. Sterile 40% (w/v) glucose solution (Baxter Healthcare Pty Ltd, NSW, Australia) warmed to body temperature was used for intra-jugular injections. Blood samples were collected at -10, -5, 5, 10, 15, 20, 30, 45, 60, and 90 min relative to glucose injection from the contralateral jugular catheter. On one occasion glucose was injected directly into the jugular vein and subsequent blood samples were collected with vacutainer tubes because the catheters were blocked. Plasma samples were prepared and analyzed for glucose and insulin.

2.3.4 Blood processing and sample analyses

All blood samples (10 ml) were collected into heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes NJ 07417-1885, U.S.A.) to prevent clotting. The samples were stored in ice and centrifuged at 4000 rpm for 15 min at 4^0 C immediately after completing the experiment. The resulting plasma was stored at -20^0 C, until analysed for plasma metabolites and hormones.

Plasma glucose (Trinder, 1969) and NEFA (Dalton and Kowalski, 1967; McCutcheon and Bauman, 1986) concentrations were measured by the glucose oxidase method and using WAKO NEFA C Kit, respectively, on a Cobas Fara II autoanalyser (Hoffman LA Roche Ltd, Switzerland). Intra- and inter-assay coefficients of variation for glucose and NEFA were 1.9% and 4.6%, 2.6% and 3.8%, respectively.

Plasma insulin and GH concentrations were determined by RIA using crystalline bovine insulin (Sigma I 5500, Lot 55F - 0536, 26.2 iu/mg Sigma Chemical Co., St Louis,

Mo., 63178, U.S.A.) and bovine GH, respectively, following the method described by Flux *et al.* (1984). The GH assay used bovine GH for iodination (USDA-bGH-I1. 3.2 iu/mg) and reference standards (USDA-bGH-B1, 1.9 iu/mg). Intra- and inter-assay coefficients of variation for GH and insulin RIA were 8.4% and 12.4%, 8.6% and 13.2%, respectively.

The measurement of plasma PRL was based on a RIA described by Van Landeghem and Van de Weil (1978) using lyophilized ovine PRL (NIADDK-oPRL-I-2, NIH, Bethesda, Md., U.S.A.) as the standard. Intra- and inter-assay coefficients of variation were 9.7% and 16.6%, respectively.

Determination of DM was done according to the method described by Harris (1970). DM digestibility was measured by determining total DM ingested and DM in the faeces. Nitrogen in the feed, faeces and urine was determined by the Kjeldahl method using a Kjeltec Auto 1030 Analyser (Kjeltec Auto, Tecator, Science and Technology [NZ] Ltd).

2.3.5 Statistics

Statistical significance between the means of treatment groups was determined using ANOVA designed to account for repeated measures. Duncan's multiple comparison range test was used to compare the means within individual treatment groups when the time and the interaction between time and treatment effects within subjects were significant. During the glucose challenges, baseline corrected area under the response curve was determined for a period up to 90 min for glucose and up to 30 min for insulin after the glucose injection. Baseline corrected values for glucose at 5 to 30 min post-injection, were used to fit a linear regression model and the resulting values for glucose clearance constant (K) were used in the ANOVA to determine the significance of differences between glucose clearance rates. Data are presented as least square means ± standard errors (±SEM). A probability value less than 0.05 was considered significant. Statistical analyses were carried out using the computer package 'SAS' (The SAS System for Windows, Version 6.11, SAS Institute Inc., Cary, NC, U.S.A.).

2.4 RESULTS

2.4.1 General effects of vagotomy

It took 24-48 h following surgery for all ewes to return to pre-surgical levels of food intake and milk production. Body temperature was normal before and after surgery.

2.4.2 Feed intake

The mean (±SE) food intake in the ADV group 48 h after surgery was 1053 ± 86 g/day. The intake was gradually but significantly ($P < 0.01$) increased during the experimental period and reached a plateau at PO day 10. The intake of SO group was parallel to the intake in the ADV group as SO animals were pair fed to the *ad libitum* intake of the ADV animals (Fig. 2.1 (a)).

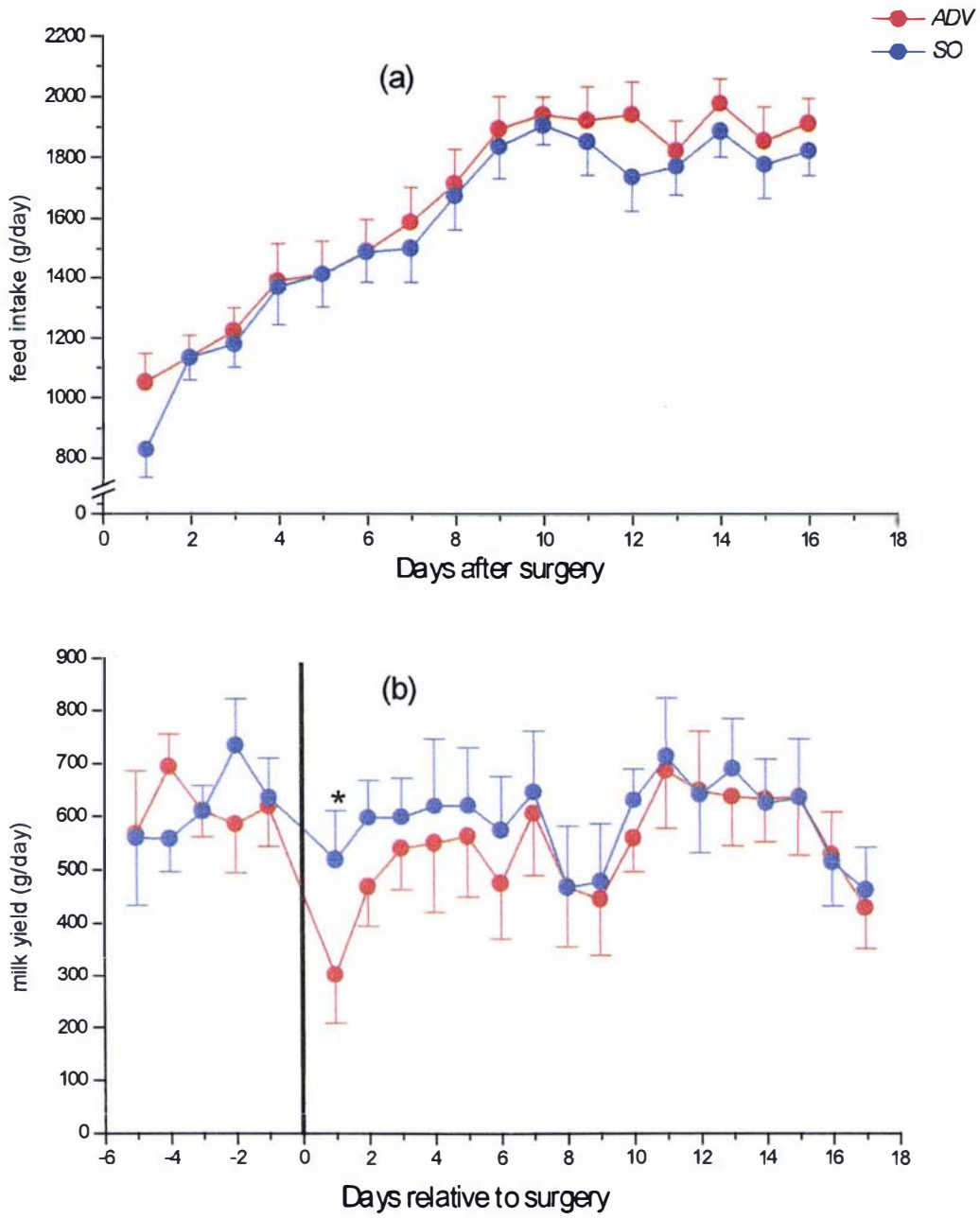

Figure 2.1 Daily feed intake (a) and milk yield (b) in vagotomized (ADV) and sham-operated (SO) lactating ewes. Pair feeding of SO ewe to the*ad libitum* intake of the ADV ewe in the pair. Thick vertical line of (b) at day 0 indicates day of surgery. Legends are shown at top right hand corner. $^{*}P < 0.05$, significance of differences between treatment groups. Vertical bars: SEM.

2.4.3 Milk yield

The milk yield was in the range of 463 ±103 to 734± 103 g/day and 430± 87 to 688 ±87 g/day in the SO and in the ADV group, respectively. The yield was not significantly different between two treatment groups. However, a day after surgery milk yield in the ADV group dropped significantly ($P < 0.05$) to 302 ± 87 g/day (Fig. 2.1 (b)).

Over the period of the trial (18 days) milk fat percentage was significantly (repeated measures analysis, $P < 0.02$) reduced by vagotomy. While on a daily basis the fat percentage in the milk from the two groups were significantly different on days 2, 3, 9, 10, 16, and 17 ($P < 0.05$) and on days 13 and 15 ($P < 0.01$) (Fig. 2.2 (a)). The milk fat yield was reduced in the ADV ewes compared with that of SO ewes (Fig. 2.2 (b)), but the overall difference was not statistically significant. Percentage of residual milk and residual milk fat in the ADV group was not significantly different from that in the SO group (data not shown).

2.4.4 Milking-associated release of hormones and metabolites

Plasma concentrations of GH, PRL and NEFA (Fig. 2.3) were not different between ADV and SO groups before and after milking on day 6 and 13.

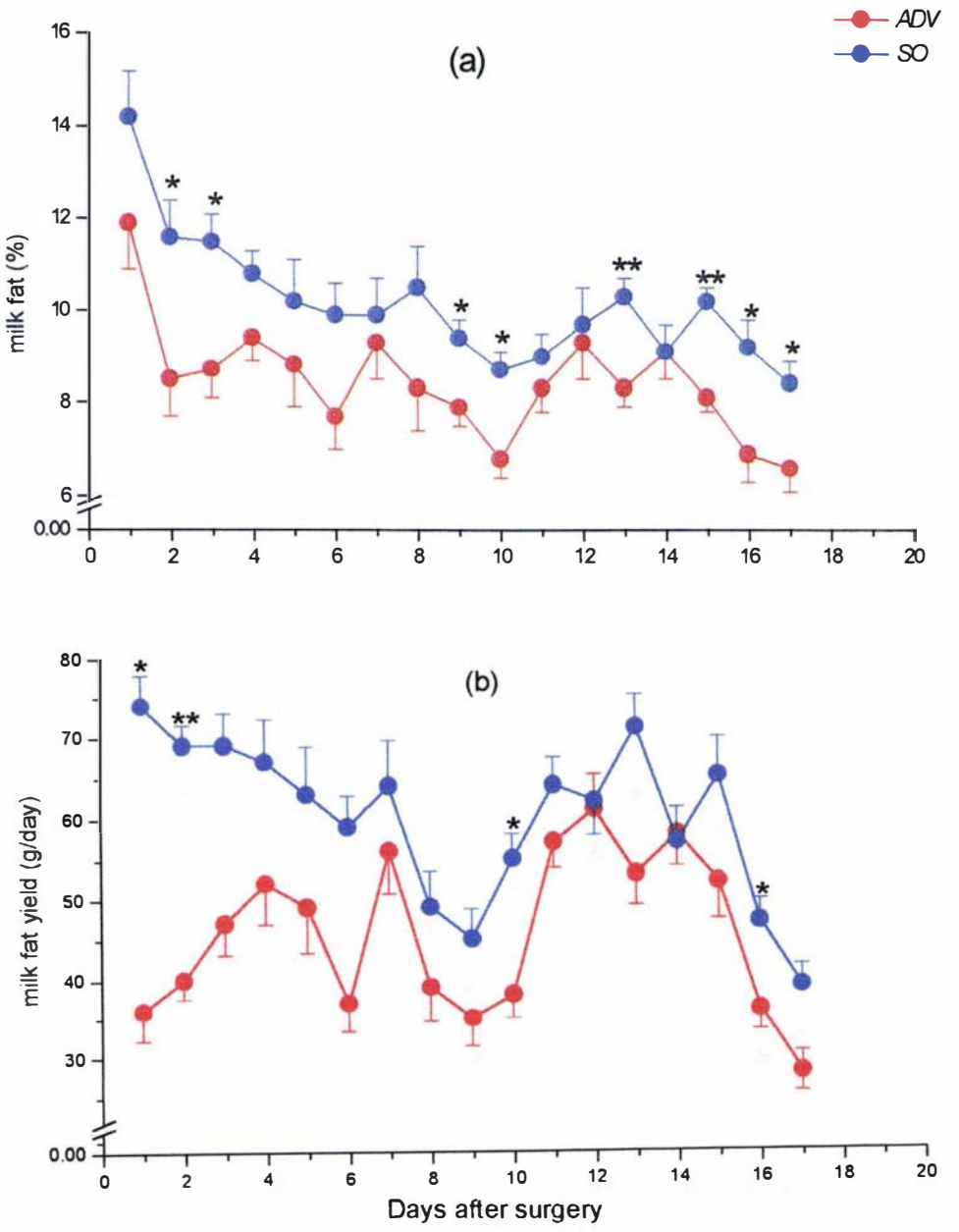

Figure 2.2 Milk fat percentage (a) and milk fat yield (b) during post-operative days 1-17 in lactating vagotomized (ADV) and sham-operated (SO) ewes. Legends are shown at top right hand corner. $^{**}P < 0.01$; $^{*}P < 0.05$, significance of differences between treatment groups. Vertical bars: SEM.

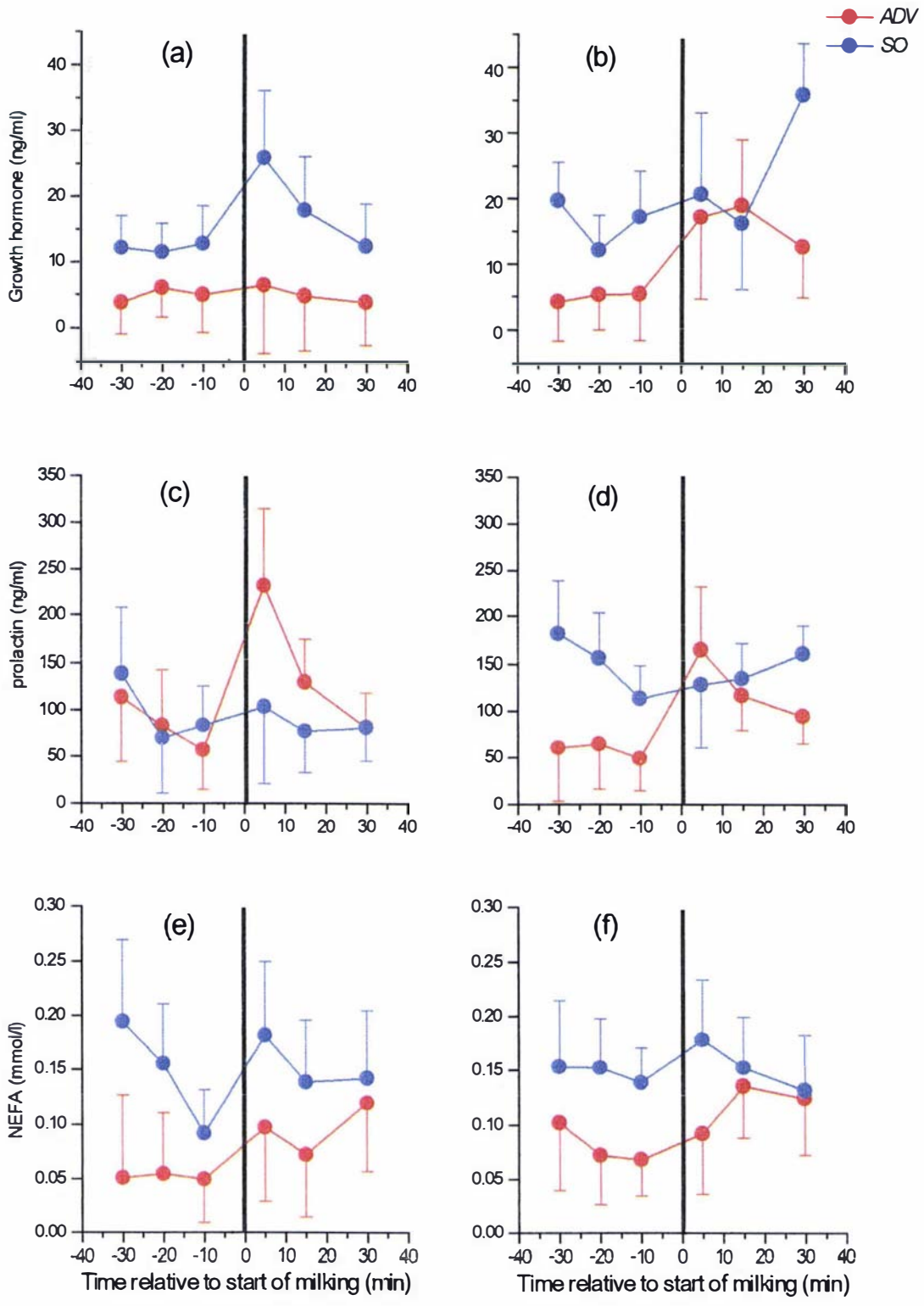

Figure 2.3 Milking-related responses of plasma concentrations of growth hormone (a), prolactin (c) and non-esterified fatty acids (NEFA) (e) at post-operative day 6 and growth hormone (b), prolactin (d) and NEFA (f) at post-operative day 13 in vagotomized (ADV) and sham-operated (SO) ewes. Legends are shown at top right hand corner. Thick vertical line at time 0 indicates time of start of milking. Vertical bars: SEM.

2.4.5 Digestibility

The mean (±SEM) DM digestibilities in the ADV and SO groups at BL-1 (53.9 ± 3.2 v. 60.1 ± 3.2, respectively) and BL-2 (51.8 ± 3.2 v. 55.3 ± 3.2, respectively) were not significantly different.

The mean (±SEM) N digestibilities in the ADV and SO groups at BL-1 (56 ± 3.5 v. 65.1 ± 3.5, respectively) and BL-2 (55.8 ± 3.5 v. 60.4 ± 3.5, respectively) were not significantly different.

The amount of N retained by ewes in the ADV group was 1.6 ± 1.7 g/day immediately after the experiment was commenced at BL-1. During this period ewes in the SO group retained 2.0 ± 1.7 g/day. Ten days after beginning of the experiment (BL-2) ewes in the ADV group retained 7.9 ± 1.7 g/day while N retention was 8.4 ± 1.7 g/day in the SO group. Significant differences in N retention were not found between the two treatment groups. However, N retention in both groups during BL-2 were significantly higher ($P < 0.05$) than that found during BL-1 and thus they were both in positive N balance during the second period.

2.4.6 Glucose challenge

2.4.6.1 Plasma glucose

Following glucose injection, differences in glucose concentrations between ADV and SO ewes were significant ($P < 0.05$) (Fig. 2.4 (a),(b)). Glucose clearance rate from the blood was slower ($P < 0.05$) in the ADV group compared to the SO group and the respective K values were 0.0403 ± 0.0112 and 0.0743 ± 0.0112. Baseline corrected total area under the curve was significantly larger ($P < 0.01$) in the ADV group than in the SO group (Fig. 2.5 (a)). Plasma glucose clearance rates were significantly ($P < 0.05$) higher following the second glucose challenge than the first glucose challenge and the respective K values were 0.0763 ± 0.0112 and 0.0383 ± 0.0112.

2.4.6.2 Plasma insulin response

During the first glucose challenge at day 7, the mean plasma insulin concentration rose from 718 ± 274 pg/ml to 3139 ± 918 pg/ml ($P < 0.05$) in the SO group. The mean

plasma insulin response in the ADV group was smaller, 1088 ± 918 pg/ml, and not significantly different from its basal concentration of 677 ± 274 pg/ml (Fig. 2.4 (c)).

At day 14, the basal plasma insulin concentrations before the second glucose challenge were 555 ± 274 pg/ml and 652 ± 274 pg/ml in the ADV group and SO group, respectively. The glucose injection increased mean plasma insulin concentration to 3787 ± 918 pg/ml ($P < 0.05$) in the SO group but only to 1973 ± 918 pg/ml in the ADV group, which was not significantly different from baseline values (Fig. 2.4 (d)). Baseline corrected total area under the insulin response curve was significantly larger ($P < 0.05$) in the SO group than in the ADV group during both challenge 1 and 2 (Fig. 2.5 (b)).

During the second glucose challenge on day 14, the plasma insulin response in the ADV group increased by 81% compared to the response during the first glucose challenge on day 7; the percentage increment of the total response in the SO group was 20%.

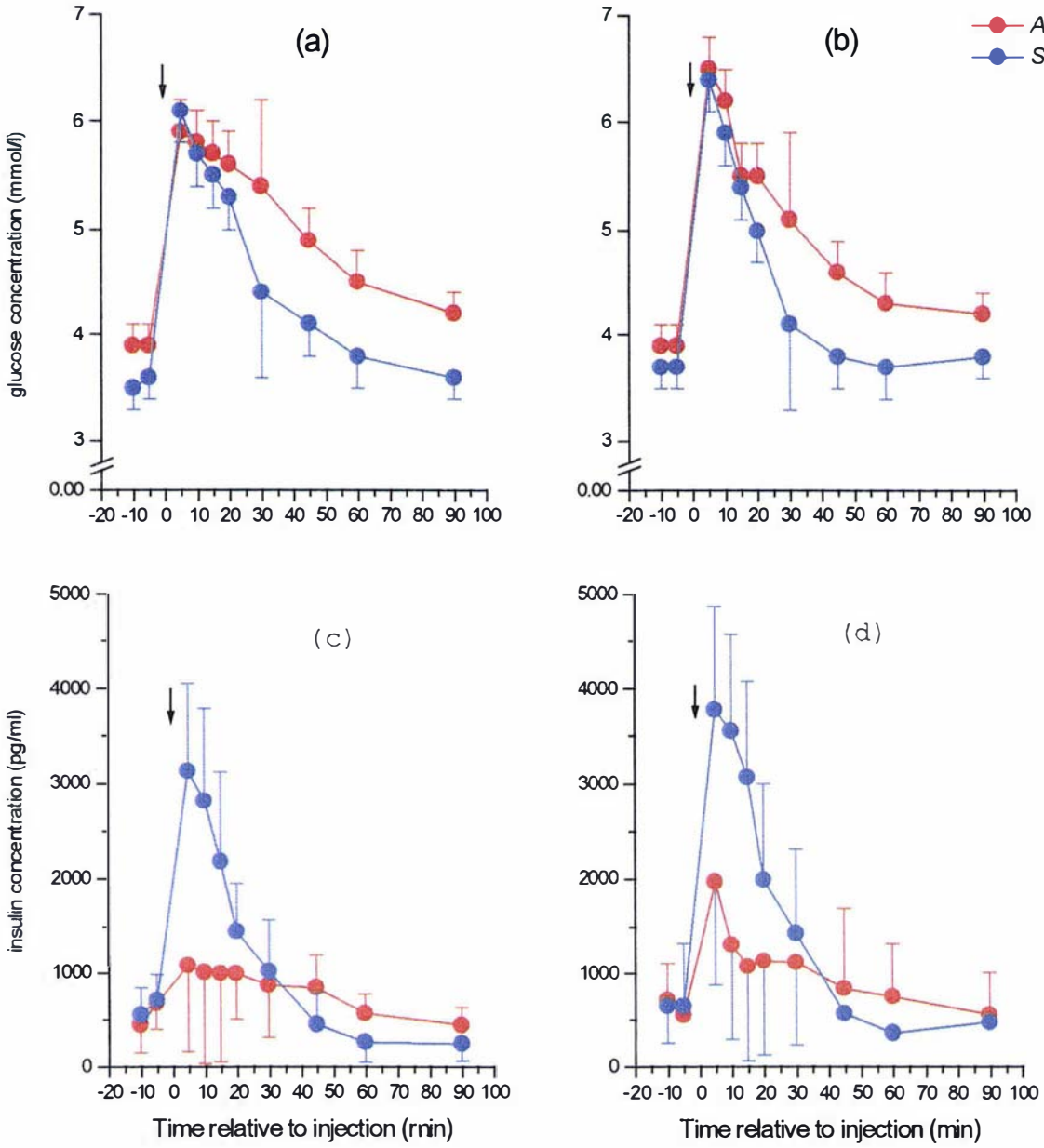

Figure 2.4 Plasma concentrations of glucose; challenge 1 (a), challenge 2 (b) and plasma concentrations of insulin; challenge 1 (c), challenge 2 (d), before and after intra-jugular glucose injection 7 days (challenge 1) and 14 days (challenge 2) after the vagotomy (ADV) or a sham-operation (SO). The arrow indicates the time of glucose injection. Legends are shown at top right hand corner. $P < 0.05$, following glucose injection, glucose or insulin concentrations between two treatment groups were significantly different at both day 7 and 14. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

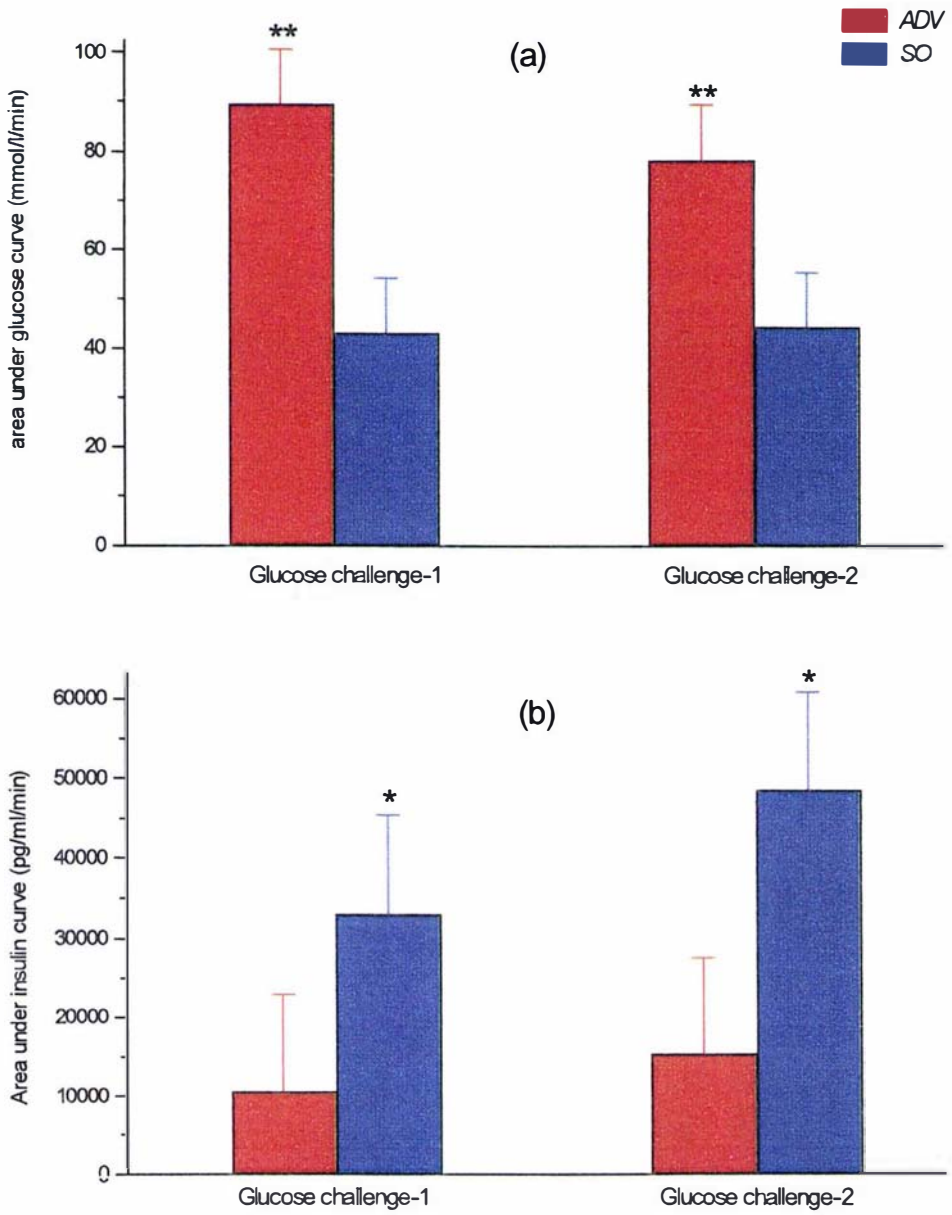

Figure 2.5 The area under the glucose response curve, corrected for baseline, for 90 min (a) and under the insulin response curve, corrected for baseline, for 30 min (b) in lactating ewes in response to an intravenous injection of glucose 7 days (Challenge 1) and 14 days (Challenge 2) after the vagotomy (ADV) or a sham-operation (SO). Legends are shown at top right hand corner. $^{*}P < 0.05$, $^{**}P < 0.01$, significance of differences between treatment groups. Vertical bars: SEM.

2.5 DISCUSSION

The present study investigated the role of the vagal innervation of just the abomasum and upper small intestine (duodenum) on milk yield and insulin response to intravenous glucose injection in lactating ewes. The absence of any long lasting effects of the surgery on food intake indicates that the surgical procedures did not cause major disruption to digestion in the selectively vagotomized or sham-operated animals.

Pair feeding was chosen to prevent differences in food intake, because of the suggestion that vagotomy depresses milk production independent of food intake (Eriksson *et al.*, 1994). That there was no overall difference in milk production suggests that vagotomy does not affect milk production but, because of the experimental design, there is still the untested possibility that vagotomy may alter production by altering food intake.

However, the differences between groups in milk yield were significant on day one after surgery and fat yield was reduced for 2 days but that these differences were not associated with differences in intake, or even a fall in intake. Milk yield and fat yield in the ADV ewes were restored by days 1 and 3, respectively. The effects on milk and fat yield were unlikely to be due to difference in energy intake but could be related to partitioning of energy or efficiency of milk removal. Both these effects could be mediated through changes in hormone concentration in the circulation.

Subdiaphragmatic vagotomy, which depresses the circulating concentrations of OT and PRL in rats, is associated with reduced milk yield (Eriksson *et al.* 1994). Eriksson *et al.* (1994) reported that litters of vagotomized rats failed to gain weight as rapidly as the control litters, indicating that vagotomized rats, in spite of eating normally, failed to give milk to their pups. It was suggested that vagotomy had interfered with the release of OT and hence milk removal, which in turn led to a reduction in milk production. We failed to demonstrate such an effect on milk yield in vagotomized ewes (Figure 2.1 (b)). In this study, however, plasma OT concentrations were not measured and two indirect indicators of efficiency of milk removal gave conflicting results. On the one hand, residual milk volume did not differ between the vagotomized and sham-operated ewes, indirectly suggesting that plasma OT concentrations were not affected by vagal denervation. It should be noted, however, that residual milk volume was measured sometime after any

effect on yield was noticed. On the other hand there was an overall depression in the fat concentration in the milk from the vagotomized ewes (Figure 2.2 (a)), which may reflect less efficient milk ejection and milk removal in this group (Gorewit and Sagi, 1984; Sagi *et al.*, 1980; Donker *et al.*, 1954).

Prolactin and GH have roles in the initiation and maintenance of lactation in ruminants (Peel and Bauman, 1987; Bauman and McCutcheon, 1986) and it was considered necessary to monitor their concentration in the present experiments even though an effect of the vagus nerve on their release has not been reported. There were no significant differences detected in basal concentrations or in milking-related concentrations of PRL and GH between the vagotomized and sham-operated ewes. It was also evident that milking did not significantly affect baseline levels of these two hormones in either of two treatment groups. Milking-associated rise of PRL concentration in cows and goats have been reported (Johke, 1969). It was reported that using more frequent blood sampling in goats, milking-induced release of PRL achieved its maximum concentrations within 4-12 min (Hart and Linzell, 1977; Hart, 1975, 1973) following milking. However, milking-associated peak PRL concentrations achieved in these experiments in goats were about 600-1000 ng/ml compared with baseline concentrations of 100-200 ng/ml, which were much higher than that observed in the present study. In contrast, Fell *et al.* (1972b) reported that in the ewe, machine milking was associated with a rapid rise in PRL concentration, but the peak levels achieved were about 150 ng/ml. These results together with the present results suggest that the ewe may not be the same as the goat or cow in PRL secretion associated with milking.

Plasma GH concentrations were not significantly different between vagotomized and sham-operated ewes before or after milking. Post-milking concentrations of GH were not different from their baseline values in either of two treatment groups. Hart (1973) did not find significant increases in GH release immediately after milking. Similarly Hart and Flux (1973) and Hart and Linzell (1977) have observed that although milking resulted in an increased GH concentration, some of the goats had low GH levels following milking. The variation in GH secretion is partly due to a larger variation among animals and partly because of the unpredictable pulsatile nature (brief period of secretion) of GH release (Tannenbaum *et al.*, 1990; Patel and Srikant, 1985). Furthermore, similar investigations

revealed no increase in GH in the blood during milking in the cow (Tucker, 1971) or suckling in the dog (Tsushima *et al.*, 1971), in which only 2 post-milking blood samples at 15 and 30 min had been obtained (Tsushima *et al.*, 1971). Therefore, it is possible that in the present study, peak concentrations of GH may have gone undetected in the control animals since only 3 blood samples during 30 min post-milking period were drawn.

Insulin secretion in response to glucose injection was significantly suppressed in the vagotomized group, which was also reflected in the significantly reduced rates of glucose removal in the vagotomized group (Figures 2.4 and 2.5). This result is consistent with the observations of Bloom and Edwards (1981) who showed that atropine completely blocked the release of insulin following an intravenous infusion of glucose in 2-3 months old calves. Furthermore, pre-treatment of calves with atropine suppressed the rise in plasma insulin concentration in response to electrical stimulation of the vagus nerve supplying the pancreas. Together these data suggest that the vagus nerve plays an important role in mediating the pancreatic insulin response during hyperglycaemia in lactating ewes and the present results are strongly supported by data from the literature (Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Singer *et al.*, 1989; Niijima, 1986; Adrian *et al.*, 1983; Bloom and Edwards, 1981, 1982; Daniel and Henderson, 1967).

However, the use of systemically administered receptor blockers does not shed light on the way in which vagal pathways are involved. This can be more readily determined by studying insulin release in response to exogenous glucose (given as either a systemic infusion or an intestinal perfusion) in selectively vagotomized animals. In the present study only the vagal supply to the abomasum, pylorus and duodenum was sectioned; the hepatic branch was left intact. Berthoud and Powley (1990) and Berthoud *et al.* (1990) have shown that the gastric, rather than the hepatic, branches play the predominant role in the vagal control of the endocrine functions of the pancreas. Although the anatomical location of the branch innervating the pancreas in ruminants is not clear, strong data from anatomical and electrophysiological studies in the literature indicated the existence of neural pathways between the stomach, duodenum and pancreas (Ami *et al.*, 1993; Kirchgessner and Gershon, 1990, 1989; Poulsen *et al.*, 1983; Matsuo and Seki, 1978; Tiscornia, 1977; Stavney *et al.*, 1963), which were discussed in detail in Section 1.4.3.1.

The results presented in this chapter are consistent with the hypothesis that severing the gastric (abomasal) and duodenal branches of the vagus nerve modified the pancreatic β-cell response to an intravenous glucose load. However, further investigations were undertaken in succeeding chapters to determine the interrelationships of the vagal network in the GI tract, brain and pancreas and plane and pattern of feeding in sheep.

The insulin response to glucose at day 14 was higher in all ewes, compared to day 7; but the increase was greatest in selectively vagotomized ewes (81% increase) compared to a 20% increase in sham-operated ewes. The increased insulin response following second glucose challenge was also reflected in the significantly increased glucose clearance rates following the challenge 2 than challenge 1. The reason for this apparent increased insulin response a week after the first glucose challenge is not known. It is possible that the greater response to the second glucose challenge was due to the increased food intake and an improved energy balance at this time in both groups. Thus the results are consistent with the concept that if the energy balance is high enough the signal the pancreatic β-cells get from the GI tract is overwhelmed by the magnitude of the stimulus that the β-cells get from the systemic circulation in the form of nutrients.

In conclusion, these results indicate that the vagal innervation of the stomach (abomasum) and upper small intestine (duodenum) modifies the pancreatic release of insulin in response to exogenous glucose in lactating ewes. However, results on milk yield were not conclusive at this stage and thus indicate that more investigation was necessary. Therefore, further experiments into whether or not the vagal innervation of the GI tract is important in insulin release both in lactating ewes and in wethers and also nutrient partitioning in lactating ewes, were undertaken.

CHAPTER 3

THE VAGAL CONTROL OF INSULIN RELEASE AND DIGESTION IN WETHERS

3.1 ABSTRACT

The role of the vagal innervation of the abomasum, pylorus and upper small intestine in the release of pancreatic insulin in response to glucose injection, on digestibility of DM and N, and baseline concentrations of insulin and gastrin was studied in wethers at two intake levels. After a 3 week acclimatization period, denervation was carried out in 10 wethers (abomasal and duodenal vagotomy; ADV); 10 wethers underwent sham-operations (SO). After surgery they were randomly assigned to 2 feeding groups; 10 wethers (ADV=5; SO=5) on a low plane of nutrition (LP) and 10 wethers (ADV=5; SO=5) on a high plane of nutrition (HP). Feed requirements at LP (1.1 x maintenance) were calculated based on mean body weights of the wethers, and HP wethers were fed *ad libitum*. Feed was offered to all wethers from an overhead constant feeder at hourly intervals for 24 h. Feed intake of HP wethers was measured daily. Seven and 14 days after the surgery, all wethers were given an intravenous injection of glucose (100 mg/kg bw) and jugular blood samples were collected for glucose and insulin determinations. Blood samples were collected on PO days 2, 4, 6, 9, 12, 15, and 20 to determine plasma concentrations of insulin, gastrin and glucose. Two x 6 day digestibility trials were carried out. In the HP group, food intake was not significantly different between ADV and SO wethers. Following glucose injection plasma glucose clearance rates were not significantly different between ADV and SO wethers on either day 7 or 14. Following glucose injection baseline corrected area under the glucose curve was significantly ($P < 0.001$) higher in the HP group than in the LP group and it was also higher ($P < 0.05$) on PO day 14 than day 7. The insulin responses after glucose injection were not different between ADV and SO wethers at either level of nutrition at both PO days 7 and 14. Insulin concentrations following glucose injection were generally higher in wethers in the HP than in the LP group, although the differences were not statistically significant. Baseline corrected area under the insulin response curve was significantly ($P < 0.05$) higher following glucose injection on PO day 14 than that on day 7. Baseline concentrations of plasma insulin, gastrin and glucose were not different between treatment groups during a 20 day sampling period. Digestibility of DM ($P < 0.05$) and N ($P < 0.01$) was increased following vagotomy although the increase in DM digestibility was less apparent in wethers fed *ad libitum*. N retention was significantly ($P < 0.0001$) higher in

wethers in the HP group than in wethers in the LP group. However, significant increases in N retention were achieved only in the ADV wethers fed *ad libitum*, compared to respective groups of SO wethers. The findings in the present experiment did not suggest that insulin release in response to glucose injection was affected by vagotomy in sheep. However, it is possible that the design of the experiment in respect to feeding pattern, may not have been appropriate, that is hourly allocations of the diet may not be sufficient to trigger neural signals from the GI tract to the pancreas. Alternatively the effect of vagotomy on insulin release in response to glucose injection may be confined to lactating ewes. The results further indicate that the magnitude of insulin release in response to exogenous glucose depends on the plane of nutrition. The vagal innervation of the GI tract is important in the control of digestion in wethers and it is hypothesised that the increased digestibility of DM and N following vagotomy is mediated through an increased secretion of SS from the GI tract, which, in turn, decreases the rate of passage of digesta allowing greater retention time in the abomasum and intestine.

3.2 INTRODUCTION

In the previous experiment with lactating ewes (Chapter 2), pancreatic insulin secretion in response to intra-jugular injection of glucose was significantly suppressed by vagotomy of the abomasum and duodenum. It was suggested that this response to cutting the vagal innervation of the GI tract might also occur in non-lactating ruminants. Furthermore, since non-lactating animals would be a more convenient model with which to study this phenomenon the effects of vagotomy were investigated in wethers.

Digestibility of DM and N was studied in the previous experiment (Chapter 2). In that study, digestibility of DM and N was not affected by vagotomy in lactating ewes. It was therefore decided to study these effects in wethers using a different feeding regimen. The measurements can also be taken more accurately since the collection of faeces and urine can be done separately in wethers.

The experiments described in this chapter were designed to investigate the effects of cutting the vagal innervation of the abomasum, pylorus and duodenum on the release of pancreatic insulin in response to intra-jugular glucose administration in wethers at two

levels of nutrition. The effect of vagotomy on digestibility of DM and N, baseline concentrations of insulin, gastrin and glucose was also studied.

3.3 MATERIALS AND METHODS

3.3.1 Animals, housing and acclimatization

Thirty Romney wethers were obtained from Massey University farms, Palmerston North. They were housed individually in metabolism crates indoors in a temperature controlled room (17-18 ^{0}C). All animals were fed lucerne pellets (12 MJ ME/kg DM) *ad libitum* and a small amount (less than 100 g/day) of lucerne chaff for the period of three weeks during which time they were accustomed to housing, handling, feeding and other routine maintenance procedures. Water was available *ad libitum;* food was offered from an overhead feeder at hourly intervals. Twenty of the animals that had adapted and were eating well after the initial 3 week period were selected for the experiment. They underwent surgery and were randomly allocated to either abomasal and duodenal vagotomized (ADV; n=10) or sham-operated (SO; n=10) groups (for details see Section 3.3.3). All procedures involving wethers were approved by the Massey University and AgResearch Animal Ethics Committee.

3.3.2 Surgical procedures

Surgery was carried out as described in Section 2.3.2.1 (see also No. 1, 2, & 3 of Figure 1.1). In the present experiment, the abomasal and pyloric branches of the dorsal vagus nerve was also sectioned (see No. 5 & 6 of Figure 1.2). Jugular catheters were inserted as described in Section 2.3.2.2.

3.3.3 Experimental design

Allocation of wethers to different treatment groups was carried out as follows. Surgery was carried out over three days. On each of the first 2 days, 8 randomly selected wethers underwent abomasal and duodenal vagotomy (ADV; n=4) or sham-operation (SO; n=4), while on day 3 the remainder underwent surgery (ADV, n=2; SO, n=2). After surgery, each treatment group, ADV-HP, ADV-LP, SO-HP and SO-LP, was allocated five animals 2 from each of the first 2 days of surgery and 1 from the third day.

3.3.3.1 Feeding

Starting immediately after surgery, a randomly selected group of ADV (n=5) and SO (n=5) wethers was fed *ad libitum* (HP) while the second group of ADV (n=5) and SO (n=5) wethers was fed at 1.1 x daily energy requirements for maintenance (LP) as estimated from body weight (Rattray, 1986). The mean (±SEM) body weights at the time of surgery were 26 ± 0.8 and 26 ± 0.7 kg in the HP group and in the LP group, respectively. The overhead feeders were adjusted to deliver one twenty-fourth of the daily ration every hour.

The amount of food ingested by each individual animal in the HP group was recorded daily. Samples of fresh lucerne pellets were stored frozen at different times for analyses of DM and N. Body weights were recorded 3 times during the experimental period.

3.3.3.2 Digestibility and nitrogen balance

Two x 6 day digestibility trials were carried out on PO days 8 to 14 (BL-1) and 15 to 21 (BL-2). Urine was collected into plastic buckets containing 200 ml of 2% HCl to prevent volatilisation of NH_3 and faeces were collected in plastic bags attached to the animals with a harness. The volume of urine and weight of faeces were recorded daily for 6 days and each day 10% sub-samples of urine and faeces were taken and stored frozen. Subsequently, the sub-samples were pooled across the 6 days for each animal and analysed for DM and N.

3.3.3.3 Baseline plasma hormones and metabolites

Blood samples were collected between 0900-1100 h on PO days 2, 4, 6, 9, 12, 15, and 20 for measurement of baseline concentrations of insulin, gastrin and glucose.

3.3.3.4 Glucose challenge

Glucose challenge experiments were carried out on PO days 7 and 14. All wethers were given a bolus injection of glucose (100 mg/kg bw) through one jugular catheter inserted 2-3 days before the challenge. Sterile 40% (w/v) glucose solution (Baxter Healthcare Pty Ltd, NSW, Australia) warmed to body temperature was used for intra-

jugular injections. Blood samples were collected at –15, -10, -5, 5, 10, 15, 20, 30, 45, 60, and 90 minutes relative to glucose injection from the contralateral jugular catheter. Harvested serum was analyzed for glucose and insulin.

3.3.4 Blood processing and sample analyses

All blood samples (10 ml) were drawn from jugular catheters and a proteinase inhibitor, Aprotinin (Lot no: 69639, ICN Biomedicals Inc., 1263, South Chillicothe Road, Aurora, Ohio, 44202, U.S.A.) was added to serum collecting tubes (Becton Dickinson vacutainer system, Rutherford, New Jersey, 07070, U.S.A.) at the rate of 100 i.u. per ml blood prior to blood sampling. All blood samples were left at room temperature for 20-30 minutes and centrifuged at 4000 rpm for 15 min. The resulting serum was stored at -20^0 C until analyzed for plasma hormones and metabolites.

Plasma concentrations of glucose and insulin were determined as described previously (Section 2.3.4). Plasma gastrin concentrations were determined in triplicate by a RIA (Simpson *et al.*, 1993) based on the method of Hansky and Cain (1969). The antibody used was Hansky's Ab74 (the generous gift of Dr Hansky). Synthetic human nsG17 (Research Plus, Bayanne, NJ, USA) was used to prepare radioactive label and standards. Intra- and inter-assay coefficients of variation recorded in the laboratory were 10% and 21%, respectively.

DM in feed and faeces and N in feed, faeces and urine were determined as described previously (Section 2.3.4).

3.3.5 Statistics

Statistical significance between the means of treatment groups was determined using ANOVA designed to account for repeated measures. During glucose challenge baseline corrected total area under the response curve was determined for 90 min and 30 min for glucose and insulin, respectively. Baseline corrected values for glucose at 5 to 30 min post-injection, were used to fit a linear regression model and the resulting values for glucose clearance constant (K) were used in the ANOVA to determine the significance of differences between glucose clearance rates. Data are presented as least square means ± standard errors (±SEM). A probability value less than 0.05 was considered significant.

Statistical analyses were carried out using the computer package 'SAS' (The SAS System for Windows, Version 6.11, SAS Institute Inc., Cary, NC, U.S.A.).

3.4 RESULTS

3.4.1 General effects of vagotomy

It took 24-48 h following surgery for all wethers to return to pre-surgical levels of food intake. Body temperature was normal before and after surgery.

3.4.2 Feed intake and body weight changes

Following surgery wethers on the LP intake ate their hourly portion of the daily ration within a few minutes at each feed. The differences in food intake between ADV and SO wethers fed *ad libitum* (HP) were not significant (Fig. 3.1 (a)).

Figure 3.1(b) shows the body weight gains of both ADV and SO wethers in the LP and HP groups. All wethers gained body weight during the experiment because they were fed above maintenance requirement but the increases were small in wethers in the LP group. When pre-surgery body weights were taken as covariates, body weight increases after surgery were significantly ($P < 0.001$) larger in the HP group than in the LP group.

3.4.3 Dry matter and nitrogen digestibility and nitrogen balance

As there was no difference in digestibility of DM and N or N retention between BL-1 (PO days 8 to 14) and BL-2 (PO days 15 to 21), data were pooled and the resulting values used in the analysis of variance. DM digestibility was significantly ($P < 0.05$) higher in the ADV group than in the SO group although it was only slightly higher in the ADV wethers fed *ad libitum* (Fig. 3.2 (a)). The effects due to feed or interaction between vagotomy, feed and balance were not significant.

The effects of vagotomy and plane of nutrition on N digestibility were highly significant ($P < 0.01$). Nitrogen digestibility was higher in the ADV group than in the SO group and it was higher in the HP group than in the LP group (Fig. 3.2 (b)). None of the interaction effects were significant.

The effect of plane of nutrition on N retention was highly significant ($P < 0.0001$). The amount of N retained by wethers in the HP group was higher than that retained by wethers in the LP group. The interaction effect between vagotomy and level of nutrition tended to be significant ($P < 0.08$) and the N retention was significantly ($P < 0.05$) higher in the ADV wethers fed *ad libitum* but only slightly higher in the ADV wethers on restricted intake, compared to SO wethers in each respective intake group (Fig. 3.2 (c)).

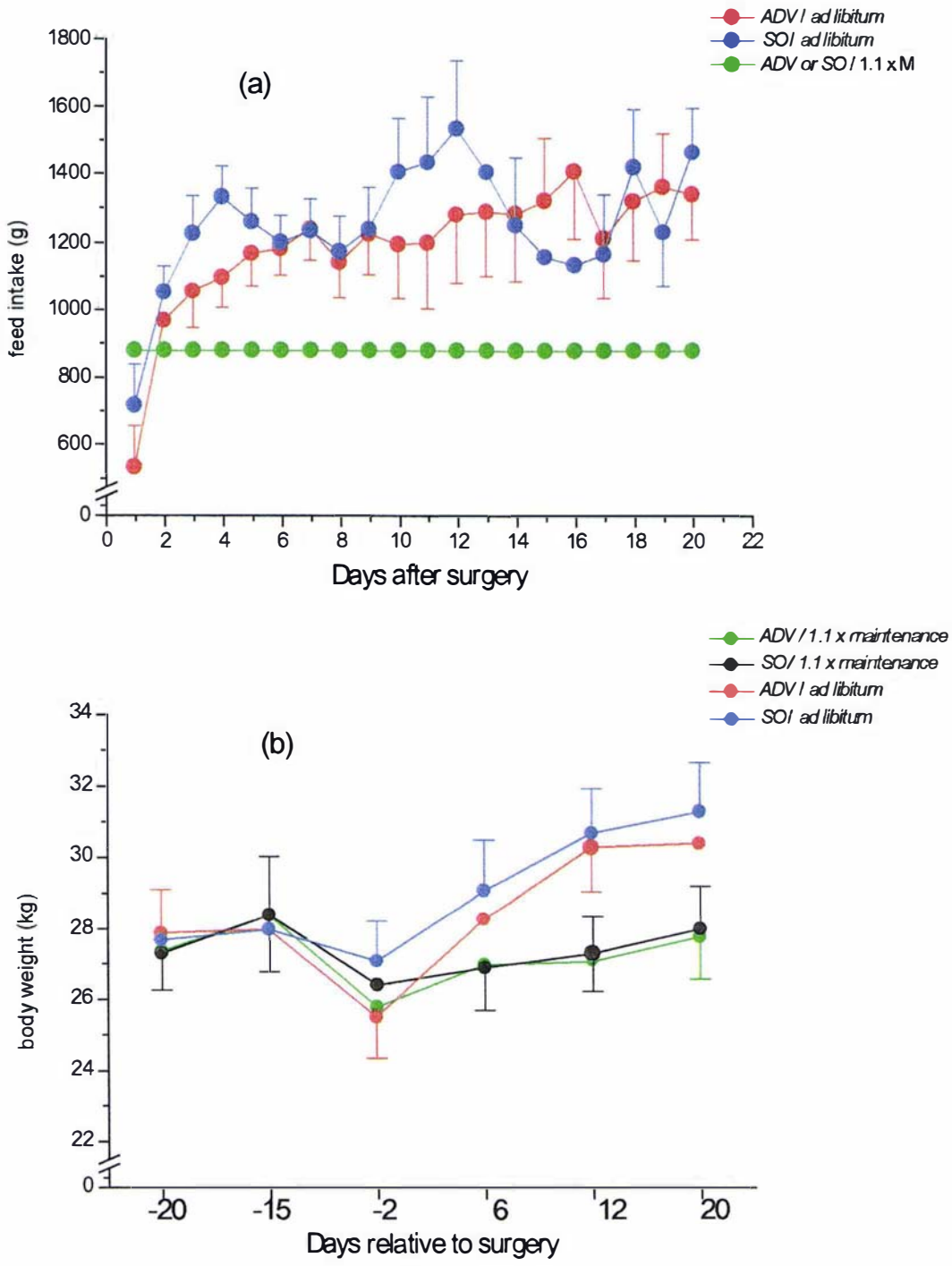

Figure 3.1 Daily feed intake (a) and body weight changes (b) in vagotomized (ADV) and sham-operated (SO) wethers fed *ad libitum* and fed at 1.1 x maintenance intake level. Legends are shown at top right hand corner. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

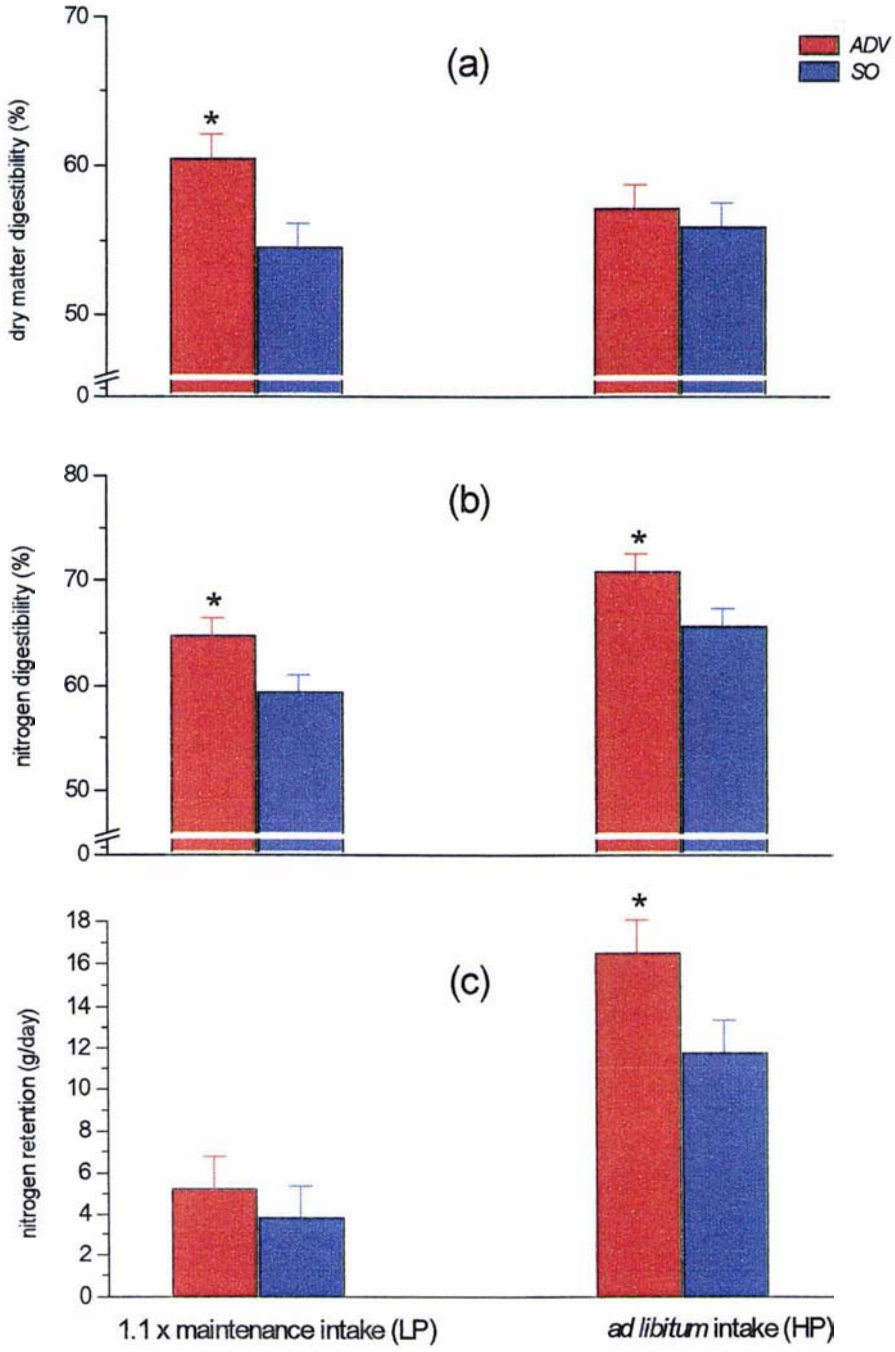

Figure 3.2 Dry matter digestibility (a), nitrogen digestibility (b), or nitrogen retention (c) in vagotomized (ADV) and sham-operated (SO) wethers fed at 1.1 x maintenance and *ad libitum*. *P < 0.05, significance of differences between treatment groups. Legend are shown at top right hand corner. Vertical bars: SEM.

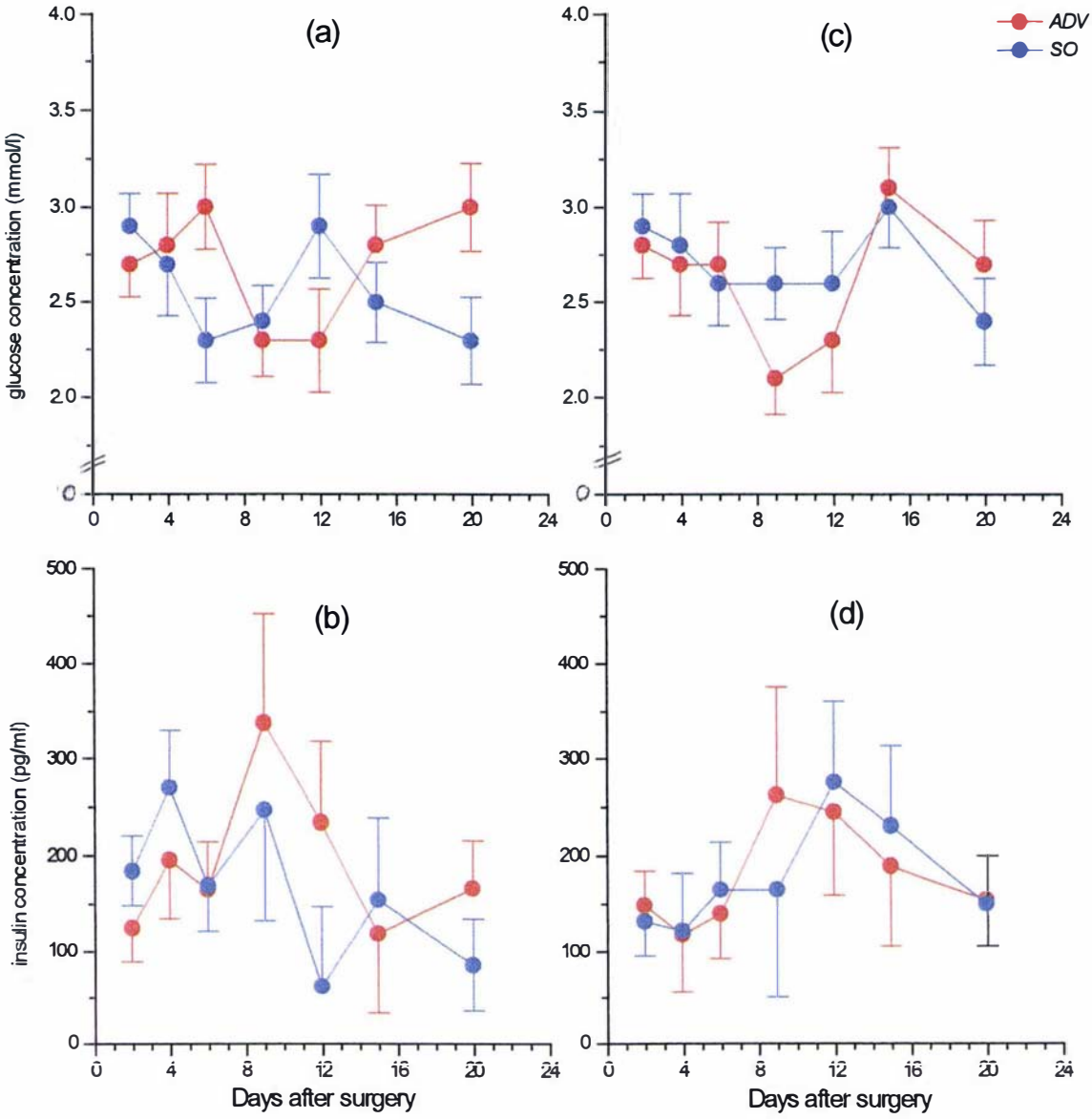

Figure 3.3 Baseline plasma concentrations of glucose (a) and insulin (b) in vagotomized (ADV) and sham-operated (SO) wethers fed at 1.1 x maintenance, and glucose (c) and insulin (d) in vagotomized (ADV) and sham-operated (SO) wethers fed *ad libitum*. Legends are shown at top right hand corner. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

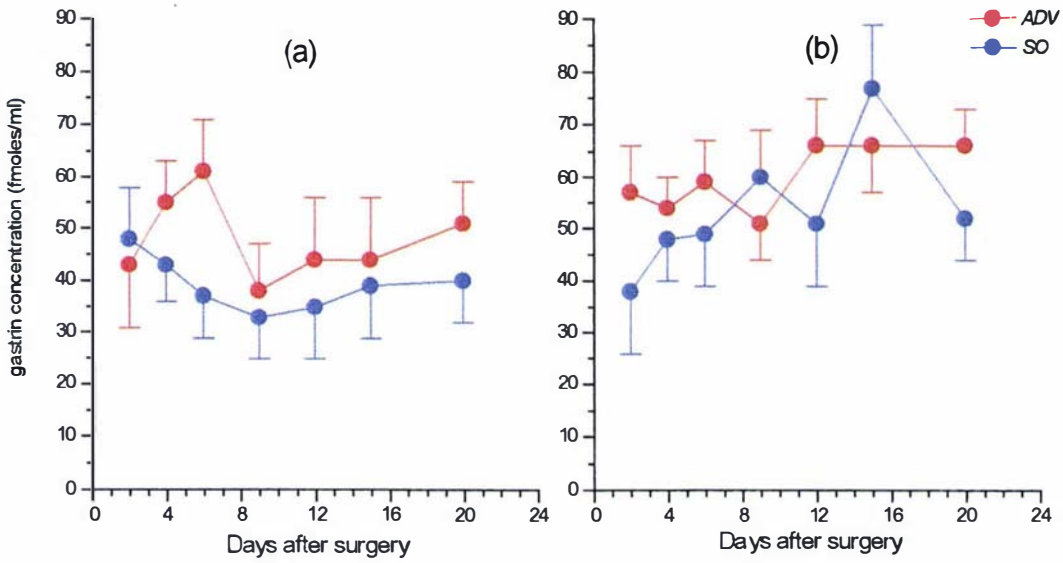

Figure 3.4 Baseline plasma concentrations of gastrin in vagotomized (ADV) and sham-operated (SO) wethers fed at 1.1 x maintenance (a) or *ad libitum* (b). Legends are shown at top right hand corner. Vertical bars: SEM.

3.4.4 Plasma hormones and metabolites

Basal concentrations of plasma glucose, insulin (Fig. 3.3) and gastrin (Fig. 3.4) were not significantly affected by either vagotomy or plane of nutrition.

3.4.5 Glucose challenge

3.4.5.1 Plasma glucose

Figure 3.5 ((a),(c)) and Figure 3.6 ((a),(c)) show plasma glucose concentrations before and after glucose injection on PO days 7 and 14, respectively. Following glucose injection plasma glucose clearance rates or area under the glucose curve, corrected for baseline, were not significantly different between the ADV and SO groups (data not shown). However, glucose clearance rates were significantly ($P < 0.001$) higher in the HP group than in the LP group and the respective K values were 0.0971 ± 0.005 and 0.0701 ± 0.005. This resulted in a significantly ($P < 0.001$) higher, baseline-corrected, total area under the glucose curve for 90 min, in the LP group than in the HP group (data not shown). Further, baseline corrected area under the glucose curve was significantly ($P < 0.05$) lower following the second glucose challenge than the first challenge (data not shown).

3.4.5.2 Plasma insulin response

Plasma insulin concentrations following glucose injection were not affected by vagotomy (Fig. 3.5 (b),(d); Fig. 3.6 (b),(d)). However, insulin concentrations following glucose injection were significantly ($P < 0.05$) higher following the second glucose challenge than the first challenge. This resulted in a significantly ($P < 0.05$) higher baseline corrected total area under the insulin curve for 30 min, for the second challenge compared with the first challenge (data not shown). Wethers fed *ad libitum* generally had a higher insulin concentrations when compared to concentrations in those fed on the low plane of nutrition, although the differences did not reach statistically significant levels.

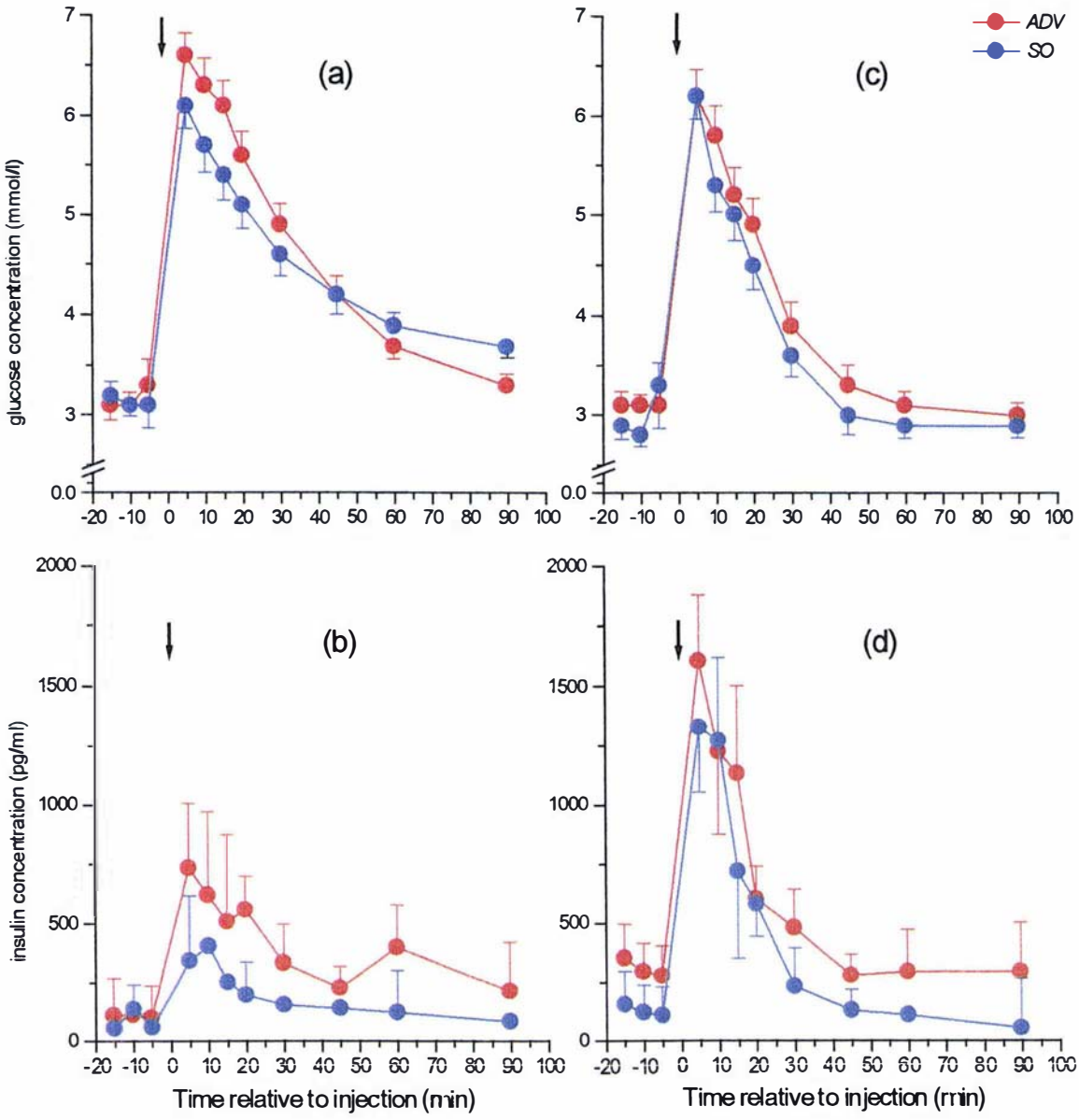

Figure 3.5 Plasma glucose and insulin concentrations in vagotomized (ADV) and sham operated (SO) wethers before and after intra-jugular glucose injection at day 7. Glucose (a) and insulin (b) in wethers fed at 1.1 x maintenance and glucose (c) and insulin (d) in wethers fed *ad libitum*. Legends are shown at top right hand corner. The arrow indicates time of glucose injection. Vertical bars: SEM.

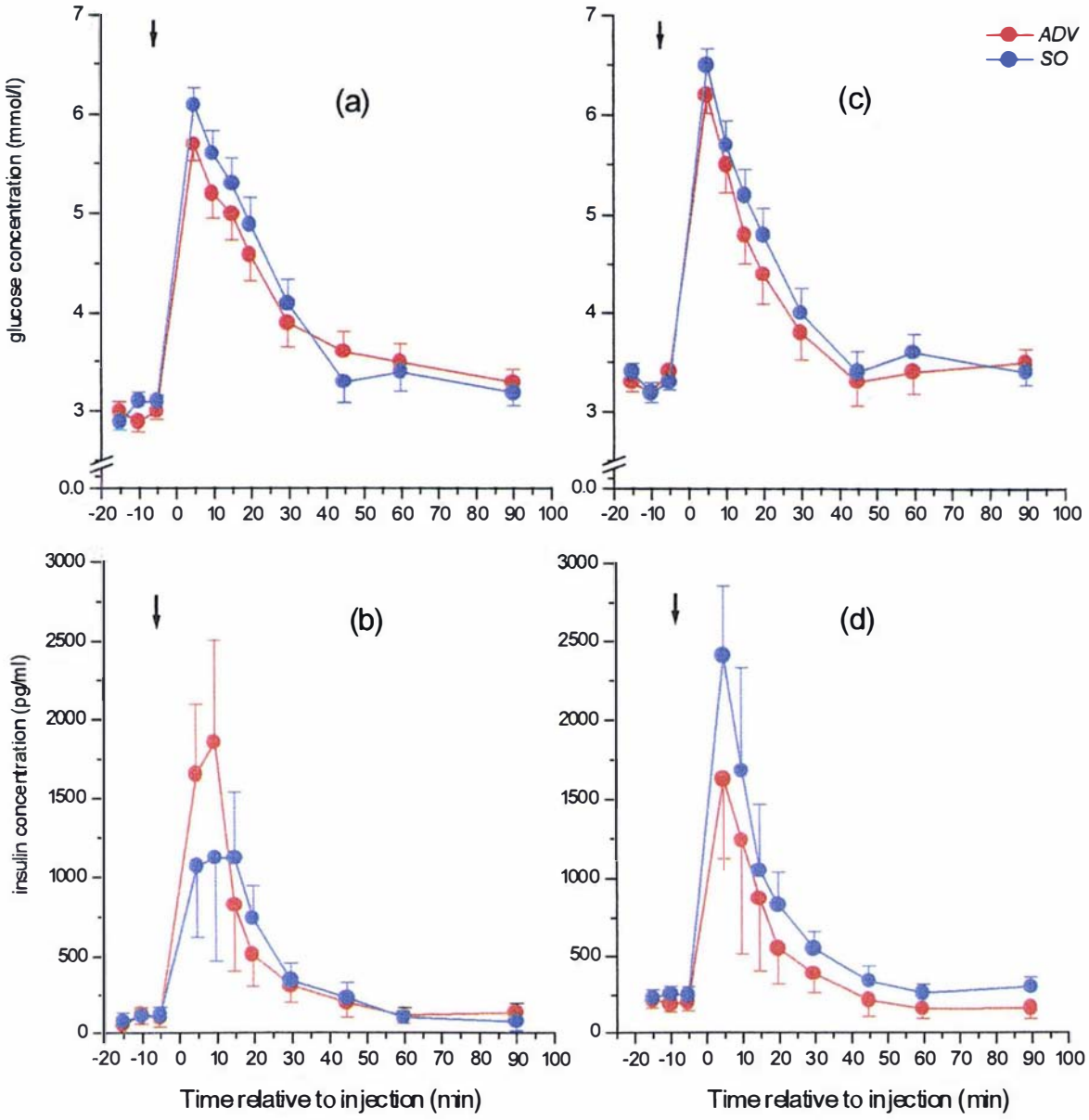

Figure 3.6 Plasma glucose and insulin concentrations in vagotomized (ADV) and sham operated (SO) wethers before and after intra-jugular glucose injection at day 14. Glucose (a) and insulin (b) in wethers fed at 1.1 x maintenance, and glucose (c) and insulin (d) in wethers fed *ad libitum*. Legends are shown at top right hand corner. The arrow indicates time of glucose injection. Vertical bars: SEM.

3.5 DISCUSSION

The present study investigated the role of the vagal innervation of the abomasum, pylorus and upper small intestine (duodenum) in the release of pancreatic insulin in response to intrajugular glucose injection, on food intake, digestibility and baseline concentrations of plasma hormones and metabolites in wethers.

There were no differences detected in baseline concentrations of plasma glucose, insulin and gastrin between the vagotomized and sham operated groups. It is possible that in this study, timing of sampling (see Section 3.3.3.3), particularly in relation to feeding and also differences in sampling time of the day, may not have been appropriate to detect baseline differences. There were significant variations in the concentration of hormones and metabolites in the blood over the day, some of which may have been influenced by vagal innervation of the GI tract. For example, Bloom *et al.* (1978) reported that administration of atropine strongly suppressed the initial rapid rise in plasma gastrin concentration during the first 10 minutes of feeding in calves. This indicates that the vagal innervation to the GI tract is important in stimulating gastrin secretion, probably in the initial phase of gastric digestion.

The increase in plasma insulin levels following glucose injection was not affected by vagotomy in wethers. It is possible that because these wethers were fed hourly, the amount of food reaching the GI tract at anytime was very small, particularly in the LP group. In contrast, in the previous experiment in lactating ewes (Chapter 2), SO ewes were fed to the *ad libitum* intake of the ADV ewes and the ration was offered twice a day. In the present experiment, insulin response following glucose injection was generally higher in wethers fed *ad libitum*. This was also reflected in the significantly increased rates of glucose removal in these animals. Therefore, it is likely that the amplitude of the vagal inputs to the pancreatic β-cells from the GI tract was dependent not only on the plane, but also the pattern, of nutrition. Thus, when the energy intake becomes very low at a given time, for example hourly feeding, the responsiveness of the β-cells to glucose injection is probably influenced to a greater extent by the degree to which β-cells are sensitized through the stimulus from the systemic circulation in the form of blood nutrients, overriding the effect of vagal inputs from the GI tract. This may have been the reason for a

lack of effect of vagotomy in animals fed hourly. Alternatively the effect of vagotomy on insulin release in response to glucose injection may be confined to lactating ewes.

The insulin response to glucose at day 14 was significantly higher in all wethers, compared to day 7. This was also reflected in the significantly increased rates of glucose removal following glucose injection on day 14. It is possible that the greater response to glucose challenge 2 was due to a higher energy balance at this time in all wethers, particularly wethers in the HP groups (Figure 3.1 (b)). Thus the results are consistent with the concept that if energy balance is high enough any neural signal the pancreas may get from the GI tract is overwhelmed by the magnitude of the stimulus that the pancreas gets from the systemic circulation in the form of nutrients. Thus the findings are consistent with results from the previous experiment in lactating ewes where the insulin response to glucose injection was higher a week after the first glucose challenge.

Following surgery the wethers fed *ad libitum* gained weight more rapidly than those fed at 1.1 x maintenance which gained weight steadily (Figure 3.1 (b)) but there was no effect of vagotomy on the rate of gain, although digestibility of food was affected by vagotomy.

The availability of nutrients for absorption is determined by a variety of factors including those that affect the efficiency of food digestion in the GI tract. Digestibility of DM and N was significantly increased in the vagotomized wethers compared to that in sham-operated wethers (Figure 3.2). The exact mechanism responsible for this increased digestibility in vagotomized wethers is not clear. The rate of movement of digesta through the GI tract is a major factor influencing how much nutrient can be obtained by the individual animal from a given diet. In ruminants, the major contractions of the rumen, reticulum and omasum are controlled via the vagus nerves of the GI tract, co-ordinated through gastric centres in the vagal nuclei of the medulla oblongata (Gregory, 1982; Harding and Leek, 1971; Stavney *et al.*, 1963; Titchen, 1958; Iggo, 1956; Habel, 1956; Duncan, 1953). It is unlikely that there was any direct change in the rate of contraction as innervation of this part of the tract was left intact in these experiments. It is possible that the increased digestibility following vagotomy might have resulted from a decreased abomasal emptying leading to an increased food retention time.

There are several ways by which vagotomy might slow abomasal emptying in sheep. There is sensory input into the gastric centres of the medulla via vagal sensory nerves innervating the abomasum and duodenum, and which reflexly affect reticulum ruminal motility and digesta flow (Titchen, 1968). It is possible that these afferent pathways that mediate the effects of acidity or abomasal distension were interrupted in the vagotomized wethers, resulting in a slower rate of digesta flow.

It is also possible that SS, a hormone secreted from the pancreas in sheep (Reddy *et al.*, 1984), is a possible candidate in the reduction of abomasal emptying in the vagotomized animals. Schusdziarra *et al.* (1979) proposed that splanchnic release of SS from the GI tract is involved in the regulation of nutrient availability for absorption in the dog. Infusion of sodium caseinate and methionine mixture into the abomasum in growing sheep reduced plasma SS concentration, suggesting that the release of SS is closely associated with the presence of nutrients in the GI tract of ruminants (Barry *et al.*, 1982). Although plasma SS concentrations were not measured in the present experiment, SS inhibits the passage of digesta through the abomasum as well as small intestine in sheep while continuous infusion of SS increases total retention times of markers in the abomasum and intestine (Faichney and Barry, 1984). This supports the findings of Bloom *et al.* (1975) that administration of SS inhibits gastric emptying in dogs. Indeed, the inhibitory effect of the vagus nerves on SS secretion has been reported in several species. Severance of the gastric vagal innervation caused an increased release of SS in lactating rats (Eriksson *et al.*, 1994). Furthermore vagal stimulation inhibited the release of SS into the portal blood in cats (Uvnas-Wallensten *et al.*, 1980). The inhibitory effect of the vagal innervation on SS release was further supported by the findings of Holst *et al.* (1981) who showed that release of SS from both the stomach and pancreas was inhibited in response to vagal stimulation in the pig. Later Holst *et al.* (1992) confirmed the findings using an isolated perfused porcine stomach that stimulation of the vagus nerve or intra-arterial infusion of a choline ester, carbachol, strongly inhibited SS secretion while the addition of atropine to the perfusate reversed the inhibition on SS secretion caused by the vagal stimulation. In calves pretreated with atropine, gastric emptying was strongly inhibited and the mean total volume collected was reduced from 1.54 to 0.22 litres over 210 minutes following feeding (Bloom *et al.*, 1978).

Thus there are strong data indicating that SS secretion is under the control of the vagal innervation to the stomach and small intestine. Thus one explanation for the increased digestibility of DM and N observed in the present experiments is a slower rate of passage in the vagotomized animals mediated by an increase in SS secretion.

However, the increased DM digestibility in vagotomized animals was more apparent in animals on restricted intake than in animals fed *ad libitum*, possibly because a larger intake of DM may have overridden the effect of reduced GI tract motility. In light of the greater N digestibility in the vagotomized animals, it is unclear why N retention in wethers on restricted intake was not significantly affected by vagotomy (Figure 3.2 (c)), although it is possible that the lower energy supply may have resulted in greater amino acid catabolism in both the restricted groups of sheep.

In conclusion, the findings in the present experiment do not indicate that modulation of insulin release in response to glucose injection is a general effect of vagotomy. The results indicate that the magnitude of insulin release in response to exogenous glucose increases with increasing plane of nutrition. However, the design of the present experiment may not have been sufficiently sensitive to detect an effect of the vagus on insulin release particularly as it seems that the pattern of nutrition may in fact be critical in sensitising pancreatic β-cells through neural signals from the GI tract. Therefore, it is possible that in the present experiment, the amount of food reaching the GI tract at any time may not have been sufficient to trigger neural signals from the GI tract to the pancreas. Alternatively the effect of vagotomy on insulin release in response to glucose injection may be confined to lactating ewes. Thus, to elucidate this, further investigation was needed and once more in lactating ewes. The vagal innervation to the GI tract is important in the digestion of DM and N in wethers. Although the mechanism by which vagus nerves influence digestion is not clear, available evidence suggests that vagally mediated reflexes alter either the reticulo-rumen motility or the secretion of SS from the stomach (abomasum) and intestine or both, which in turn acts on the passage of digesta through the GI tract to bring about enhanced DM and N digestibility.

CHAPTER 4

THE VAGAL CONTROL OF INSULIN RELEASE AND MILK YIELD IN LACTATING EWES: SUCKLING-ASSOCIATED OXYTOCIN RELEASE

4.1 ABSTRACT

The role of the vagal innervation of the abomasum, pylorus and duodenum on milk yield and composition, baseline concentrations of hormones and metabolites, suckling-associated OT release and in the release of pancreatic insulin in response to glucose injection and electrical stimulation of the cervical vagus nerves was studied in lactating ewes. Abomasal and duodenal vagotomy was carried out in 10 ewes (ADV); 10 ewes underwent sham-operations (SO). After surgery they were randomly assigned to 2 feeding groups; 10 ewes (ADV=5; SO=5) on a low plane of nutrition (LP) and 9 ewes (ADV=5; SO=4; one sham-operated ewe was removed from the experiments due to the development of clinical mastitis) on a high plane of nutrition (HP). Feed requirements at LP (1.3 x maintenance) and HP intake levels (2.0 x maintenance) were calculated according to their individual body weights. Food was offered twice a day at 0800 and 1700 h. Ewes were milked once a week. On these days, lambs were removed 2 h before first milking. Milk was completely evacuated from the udder immediately after i.v. injection of OT (1 i.u.). Six hours later ewes were milked following a similar routine; the volume recorded and milk yield calculated from the volume recorded and the time interval between the 2 milkings. Blood samples were collected on post-operative (PO) days 2, 4, 6, 9, 12, 15, 20, 25 and 29 to determine baseline plasma concentrations of hormones and metabolites. Suckling-associated OT release with or without simultaneous feeding was measured during PO days 19-22. Twelve and 28 days after the surgery, all ewes were given an intravenous injection of glucose (100 mg/kg bw) and jugular blood samples were collected for glucose and insulin determinations. A terminal acute experiment was undertaken in randomly selected ADV (n=3) and SO (n=3) ewes to confirm the vagotomies and also to study the effect of insulin release in response to an electrical stimulation of the peripheral ends of the cut cervical vagus nerves. Milk yield was not significantly different between ADV and SO ewes but was significantly ($P < 0.05$) higher in ewes fed on the HP than in ewes fed on the LP intake. Baseline concentrations of plasma insulin was significantly ($P < 0.05$) lower in the ADV than in the SO ewes across the 29 day sampling period. OT concentrations were significantly ($P < 0.01$) lower in the ADV than in the SO groups, although baseline corrected area under the OT response curve during suckling was not significantly different. The effect of feeding did not have a

significant effect on suckling-induced OT release. Insulin concentrations following glucose injection were not statistically significantly ($P < 0.08$) reduced in the ADV than in the SO ewes. Following electrical stimulation of the cervical vagus nerves reticular contractions were prominent in both the ADV and SO ewe but the contractions of the abomasum and duodenum were absent in the ADV ewe. Insulin release in response to electrical stimulation was significantly ($P < 0.05$) reduced in the ADV than in the SO ewes and there was no statistical indication of laterality of the vagus nerves in stimulating insulin release. The inability to demonstrate the effect of vagotomy on insulin release in response to glucose injection in lactating ewes was unexpected since the vagotomy suppressed insulin release in lactating ewes of the previous study. However, it is possible that time of glucose injection relative to feeding is probably important to get any effect of vagotomy on insulin release as the presence of food in the GI tract may be crucial for sensitizing the pancreatic β-cells through vagal inputs. However, the results of electrical stimulation of the cervical vagus nerves support the idea that release of insulin is modulated through the vagus nerves in lactating ewes and the results of the abomasal contractions confirm the vagotomies performed were complete. Results indicate that basal OT release is modulated by the vagus nerves in lactating ewes. However, milk yield was not affected by vagotomy. One possible explanation is that OT injected before each milking would have emptied the alveoli and prevented the inhibition of milk ejection and the expression of any autocrine mechanisms. Alternatively, because of the marked difference in the mammary anatomy of ewe from that of rat, a change in the yield due to a reduced OT concentration may have gone undetected.

4.2 INTRODUCTION

The release of pancreatic insulin in response to intra-jugular injection of glucose was suppressed in lactating vagotomized ewes (Chapter 2). It was suggested that this was a general effect to cutting the vagal innervation that would also occur in non-lactating ruminants. Therefore, because they would be a more convenient model the effects of vagotomy were next studied in greater detail in wethers. However, the results from this experiment did not support the above hypothesis (Chapter 3). This could have been due to problems with the design of the experiment. The wethers were fed hourly which may have

caused problems because the amount of food that had passed through the abomasum and upper small intestine was quantitatively low at any time and therefore may not have been sufficient to activate vagal signals from the GI tract to the pancreatic β-cells. On the other hand, it is also possible that the effect of vagotomy on insulin release may not be a general response and may be confined to lactating ewes (Section 3.5 of Chapter 3). Therefore, it was decided to test the effect of vagotomy once more in lactating ewes using an experimental design that would also explore in detail the effects of different planes of nutrition on the response.

It is well known that suckling causes the release of pituitary hormones such as OT and PRL in several species such as rabbits, guinea pigs, rats, sows and dogs as well as women (Tay *et al.*, 1996; Algers *et al.*, 1991; Eriksson *et al.*, 1987; Uvnas-Moberg *et al.*, 1985; Fuchs *et al.*, 1984; Robinson and Jones, 1982; Voloshin and Tramezzani, 1979). Suckling is a primary source of teat stimulation that through reflex activation causes the release of pituitary OT (Yokoyama *et al.*, 1994; Eriksson and Uvnas-Moberg, 1990; Uvnas-Moberg *et al.*, 1984; Folley and Knaggs, 1966). Feeding has been reported to enhance OT release in dairy cows, dogs, sows and rats (Svennersten *et al.*, 1990; Uvnas-Moberg *et al.*, 1985; Verbalis *et al.*, 1986). Oxytocin is essential for milk ejection in many species. The mechanisms regulating milk secretion and milk ejection have been investigated (Grosvenor and Mena, 1974). However, the mechanism(s) involving the vagus nerves in the release of pituitary OT during suckling has been investigated only in monogastric animals, for example rats. The modification of the suckling-induced release of hormones such as OT and PRL from the pituitary by vagotomy in rats (Eriksson *et al.*, 1994; Uvnas-Moberg, 1992; Uvnas-Moberg, 1989), suggest that the vagus nerves play a role in coordinating neural inputs from the GI tract, brain and the mammary gland in the release of pituitary hormones during suckling in this species and probably in lactating ruminants. However, a relationship between the vagus nerve and the release of pituitary OT during suckling with or without simultaneous feeding, has not been investigated in lactating ruminants.

In order to confirm the effectiveness of vagotomy in these experiments and to demonstrate which parts of the GI tract and associated organs were affected, an independent qualitative test was necessary. It is well known that the major contractions of

the forestomach in ruminants are controlled via the vagus nerves from the vagal nuclei in the medulla oblongata (Cottrell, 1994; Gregory, 1982; Iggo, 1956; Duncan, 1953; Stavney *et al.*, 1963). Furthermore release of pancreatic insulin in response to stimulation of the peripheral ends of the cervical vagus nerves has been reported in a variety of species including sheep (Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Pierzynowski *et al.*, 1986; Adrian *et al.*, 1983; Bloom and Edwards, 1981; Holst *et al.*, 1981; Daniel and Henderson, 1967). Therefore, a terminal acute experiment in which contractions of the stomach and duodenum and insulin release in response to electrical stimulation of the cervical vagus nerves were studied in anaesthetized ewes to provide physiological evidence to confirm the effectiveness of the vagotomy.

The experiments described in this chapter were designed to demonstrate the effects of cutting the vagal innervation to the abomasum, pylorus and duodenum on milk yield, baseline concentrations of hormones and metabolites and also pancreatic insulin release in response to intra-jugular glucose administration, at two levels of feeding. The effect of vagal denervation on suckling-associated OT release and, insulin release in response to electrical stimulation of the vagus nerves was also investigated.

4.3 MATERIALS AND METHODS

4.3.1 Animals, housing and acclimatisation

Twenty eight pregnant Romney ewes were obtained from Massey University farms, Palmerston North. They were grazed on pasture until lambing but were trained to also eat lucerne pellets. This was done by allowing them to eat pellets when brought indoors each night. After lambing they were housed indoors, each with a single lamb, in metabolism cages. One of each twin born lamb was fostered. Lambs were returned to pasture at weaning.

Ewes were accustomed to housing, handling, feeding and other routine procedures and given water *ad libitum* at all times. They were fed lucerne pellets (12 MJ ME/kg DM) *ad libitum* and a small amount (less than 100 g/day) of lucerne chaff over a two weeks acclimatisation period. Food was offered twice a day at 0800 and 1700 h. Twenty of the ewes that adapted and were eating well were selected for the subsequent experiment. All

animals underwent surgery and were randomly allocated to either abomasal and duodenal vagotomized (ADV; n=10) or sham-operated (SO; n=10) groups (for details see Section 4.3.3). All procedures involving ewes were approved by the Massey University and AgResearch Animal Ethics Committee.

4.3.2 Surgical procedures

Surgery was carried out as described in Section 2.3.2.1 (see also No. 1, 2 & 3 of Figure 1.1). In the present experiment, the abomasal and pyloric branches of the dorsal vagus nerve were also sectioned (see No. 5 & 6 of Figure 1.2). Jugular catheters were inserted as described in Section 2.3.2.2.

4.3.3 Experimental design

Allocation of ewes into different treatment groups was carried out as follows. Surgery was carried out over three days. On each of the first 2 days, 8 randomly selected ewes underwent abomasal and duodenal vagotomy (ADV; n=4) or sham-operations (SO; n=4), while on day 3 the remainder underwent surgery (ADV, n=2; SO, n=2). After surgery, ewes were allocated to one of two planes of nutrition, 1.3 x daily maintenance (LP) and 2.0 x daily maintenance (HP). Each treatment group, ADV-HP, ADV-LP, SO-HP and SO-LP, was allocated five animals 2 from each of the first 2 days of surgery and 1 from the third day.

4.3.3.1 Feeding

Starting immediately after surgery, all animals were fed *ad libitum* for 4 days. After this period a randomly selected group of ADV (n=5) and SO (n=5) ewes was fed initially at 1.1 x and then at 1.3 x daily energy requirements for maintenance (LP) while the second group of ADV (n=5) and SO (n=5) ewes was fed initially at 1.8 x and then 2.0 x daily energy requirements for maintenance (HP) as estimated from body weight (Rattray, 1986). The mean (±SEM) body weights at the time of surgery were 48.2 ± 2.5, 53.1 ± 2.5, 50.3 ± 2.5, and 49.3 ± 2.8 kg in the ADV and SO of LP group and in the ADV and SO of HP group, respectively. Body weights were recorded approximately at weekly intervals.

4.3.3.2 Milk yield and composition

Milk yield was measured once a week by the OT method. Lambs were allowed to suckle their mothers freely during the experimental period except the days during which milk volume was measured. On these days lambs were removed from their mothers 2 h before first milking. Ewes were given an intravenous injection of OT (1 i.u.) followed by complete evacuation of milk from the udder using a milking machine. Six hours later ewes were milked following a similar routine and the volume recorded. Milk protein, fat, and lactose were measured in sub-samples of milk. When milking was completed lambs were brought back to mothers. Daily milk yield was calculated from the volume recorded and the time interval between the two milkings.

4.3.3.3 Baseline plasma hormones and metabolites

Blood samples (10 ml) were collected between 1000 and 1100 h on PO days 2, 4, 6, 9, 12, 15, 20, 25 and 29 for measurement of baseline concentrations of insulin, gastrin, OT, PRL, glucose, and β-hydroxybutyrate.

4.3.3.4 Suckling-associated oxytocin release

The experiment was carried out on PO days 19 to 22 in ewes (ADV=5; SO=5) randomly selected from the LP group.

a) Suckling without simultaneous feeding

Lambs were separated from their mothers for 2 h. Two blood samples (5 ml) were taken 10 and 5 min before the return of the lambs (i.e. -10 and –5 min relative to suckling) to measure baseline concentrations of OT. Lambs were then brought back to their mothers and allowed to suckle. A blood sample was taken immediately after lambs were brought in (i.e. at the start of suckling) followed by 3 blood samples (5 ml) at 2, 5, and 10 min relative to the commencement of suckling.

b) Suckling with simultaneous feeding

The same procedure, as used for the previous experiment, was repeated but the ewes were offered some lucerne pellets at the same time the lambs were brought in to the pens and they were allowed to eat while nursing their lambs.

4.3.3.5 Glucose challenge

Glucose challenge experiments were carried out on PO days 12 and 28. All ewes were given a bolus injection of glucose (100 mg/kg bw) through one jugular catheter inserted 2-3 days before the challenge. Sterile 40% (w/v) glucose solution (Baxter Healthcare Pty Ltd, NSW, Australia) warmed to body temperature was used for intra-jugular injections. Blood samples (10 ml) were collected at –15, -10, -5, 5, 10, 15, 20, 30, 45, 60, and 90 minutes relative to glucose injection from the contralateral jugular catheter. Harvested serum was analyzed for glucose and insulin.

4.3.3.6 Physiological confirmation of vagotomy

Preparatory surgery

On PO days 49 to 54, an acute experiment, during which both cervical vagi were electrically stimulated, were carried out on randomly selected sheep to provide physiological evidence for the effectiveness of the vagotomies. Food was withheld for at least 12-14 h before the experiment. The animals were anaesthetised with at 0.5 ml/kg bw Sodium pentobarbitone (Nembutal; 60 mg/ml, Techvet Laboratories Ltd, Penrose, Auckland, N.Z.) injected intravenously. Polyethylene catheters (ID – 1.5 mm ; OD - 2.0 mm) were inserted into one femoral artery and both femoral veins and pushed deep into the vessels so that their tips lay in the abdominal aorta and the inferior vena cava, respectively. The arterial catheter was filled with heparinized saline (100 i.u./ml of 0.9% NaCl) and used to continuously monitor the aortic blood pressure using a pressure transducer (Spetramed Medical Products Pvt Ltd, Singapore) connected to a chart recorder (Model No. 2400S, Gould Instruments, U.S.A.). One of the venous catheters was used to continuously infuse barbiturate (0.06% solution of Nembutal at 1 drop/6 sec) to maintain anaesthesia and the other for blood sampling.

A ventral midline incision was made in the neck and the trachea cannulated to maintain the airways clear. Through a separate skin incision about 6-7 cm caudal to the cut end of trachea, the muscles on either side of the neck were incised and both the right and left vagus nerves were located. They were separated from the accompanying sympathetic nerve trunks, ligated and cut. The peripheral ends of the cut nerves were

connected to an electric stimulator (Neurolog System, Serial No. NL800-525, Digitimer Ltd., Welwyn Garden City, Hertfordshire, England). The dissected tissues, including the cut ends of the nerves, were kept moist with 0.9% saline warmed to 39°C.

A ventral midline laparotomy incision, approximately 20 cm long, was made to expose the abdominal viscera. Water filled balloons connected to polyethylene tubes (ID - 2 mm; OD - 2.5 mm) and pressure transducers, were positioned in the reticulum, abomasum, and duodenum to measure changes in pressure within the gut lumen. The balloons were introduced into the lumen through 1-2 cm long incisions in the gut wall and held in place with purse string sutures. The balloons in the reticulum, abomasum, and duodenum were filled with 20-25, 7.5-10, and 2.5-5 ml of water, respectively. Pressure changes were recorded using pressure transducers connected to an amplifier (Neurolog System, Serial No. NL900-424, Digitimer Ltd., Welwyn Garden City, Hertfordshire, England) and the chart recorder.

Sampling

The experiment was carried out on 6 ewes (ADV=3; SO=3), randomly selected from the LP group. Right and left cervical vagus nerves were cut and the peripheral ends stimulated separately using a standard physiological stimulus (short bursts of 1 sec duration every 10 sec at 10 mA and 40 Hz), which was applied continuously for a period of 10 min. Blood samples (10 ml) were collected at -10, -5, 0, 2, 4, 6, 8, 10, 12, 15, 20, 30 and 40 min relative to start of stimulation for measurement of plasma insulin only.

4.3.4 Blood processing and sample analyses

All blood samples were drawn from jugular catheters and a proteinase inhibitor, Aprotinin (Lot no: 69639, ICN Biomedicals Inc., 1263, South Chillicothe Road, Aurora, Ohio, 44202, U.S.A.) was added to serum collecting tubes (Becton Dickinson vacutainer system, Rutherford, New Jersey, 07070, U.S.A.) at the rate of 100 i.u. per ml blood prior to blood sampling. All blood samples were left at room temperature for 20-30 minutes and centrifuged at 4000 rpm for 15 min. The resulting serum was stored at -20° C until analyzed for plasma hormones and metabolites.

Plasma concentrations of glucose and insulin were determined as described previously (Section 2.3.4). Plasma gastrin concentrations were determined as described previously (Section 3.3.4).

Oxytocin concentration was determined after extraction using Sep-Pak-Plus C_{18} Cartridge as described in the appendix 1. Intra- and inter-assay coefficients of variation were 8.4% and 12.4%, respectively. Oxytocin results were not corrected for incomplete recovery since only the comparisons between treatment groups were made.

4.3.5 Statistics

Statistical significance between the means of treatment groups was determined using ANOVA designed to account for repeated measures. Duncan's multiple comparison range test was used to compare the means within individual treatment groups when the time and the interaction between time and treatment effects within subjects were significant. During the glucose challenges, baseline corrected area under the response curve was determined for a period up to 90 min for glucose and up to 30 min for insulin after the glucose injection, and for OT, baseline corrected area under the response curve was determined for 15 min after suckling began. For glucose challenges, baseline corrected values for glucose at 5 to 30 min post-injection, were used to fit a linear regression model and the resulting values for glucose clearance constant (K) were used in the ANOVA to determine the significance of differences between glucose clearance rates. Data are presented as least square means ± standard errors (±SEM). A probability value less than 0.05 was considered significant. Statistical analyses were carried out using the computer package 'SAS' (The SAS System for Windows, Version 6.11, SAS Institute Inc., Cary, NC, U.S.A.).

4.4 RESULTS

4.4.1 General effects of vagotomy

It took 24-48 h following surgery for all ewes to return to pre-surgical levels of food intake. Body temperature was normal before and after surgery. One sham-operated animal was removed from the experiments due to the development of clinical mastitis.

4.4.2 Feed intake and body weight

When the experiment was commenced 4 days after surgery ewes on both the LP and HP intake ate their entire ration within 24 h (Fig. 4.1 (a)). Figure 4.1 (b) shows the body weight changes in both ADV and SO ewes in the LP and HP groups. Body weights of both ADV and SO ewes in the LP group had fallen significantly ($P < 0.05$) at day 7 post-surgery when compared to body weight pre-surgery. For the ewes of the HP group, decreases of body weights were not significant. When pre-surgery body weight was taken as a covariate, body weight of the ewes in the HP group was significantly ($P < 0.001$) higher than that in the ewes of the LP group. Differences in body weight between ADV and SO ewes were not significantly different at either level of nutrition.

Body weights of the lambs were gradually increased over the period of 7 weeks (Fig. 4.2 (a)). Although body weights of lambs whose mothers fed at HP intake level were higher than that of lambs whose mothers fed at LP intake level, differences were not statistically significant ($P < 0.12$). Body weights of lambs of ADV and SO mothers fed at LP were significantly ($P < 0.05$) increased at 6 and 7 weeks of age when compared to their weights at 3 weeks of age. At 6 and 7 weeks of age, body weights of lambs of ADV mothers fed at HP were significantly ($P < 0.05$) higher compared to their weights at 3 weeks of age, however, the same weight comparison for lambs of SO mothers at the same feeding level was highly significant ($P < 0.01$).

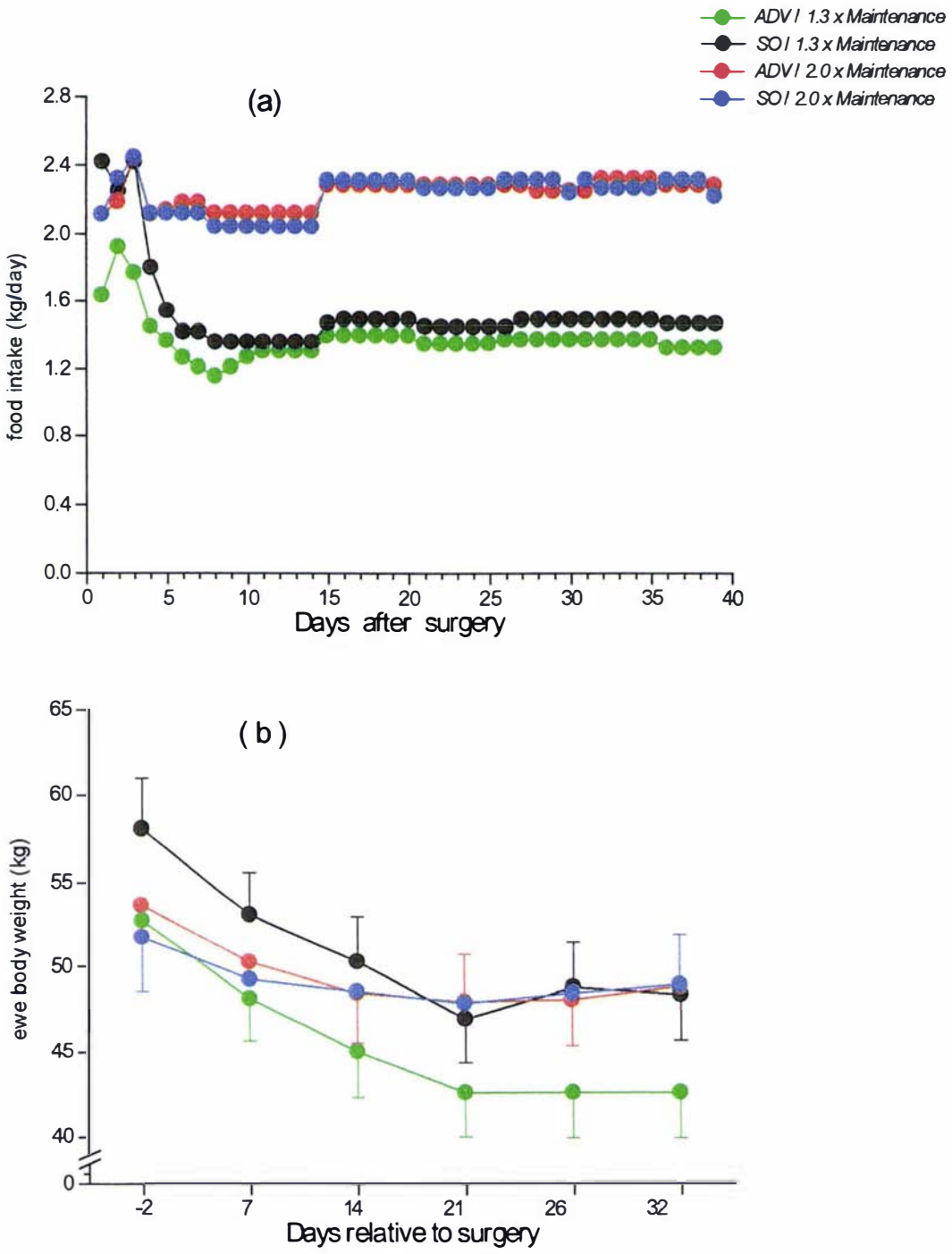

Figure 4.1 Daily feed intake (a) and body weight changes (b) in vagotomized (ADV) and sham-operated (SO) ewes fed at 1.3 x maintenance and 2.0 x maintenance intake. Legends are shown at top right hand corner. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

4.4.3. Milk yield and composition

Milk yield was significantly ($P < 0.01$) higher in ewes in the HP group than those in the LP group (Fig. 4.2 (b)). Differences in milk yield between ADV and SO ewes were not significantly different at either level of nutrition. Milk yields fell by the fourth milking (i.e. at about 6 weeks of lactation) and decreased further by the fifth milking (i.e. at about 7 weeks of lactation) (Fig. 4.2 (b)).

Yields of milk fat (Fig. 4.3 (a)) and lactose ($P < 0.05$) (Fig. 4.3 (b)) and protein ($P < 0.001$) (Fig. 4.3 (c)) were higher in the ewes of HP than in the ewes of LP group but not different between ADV and SO ewes at either level of nutrition.

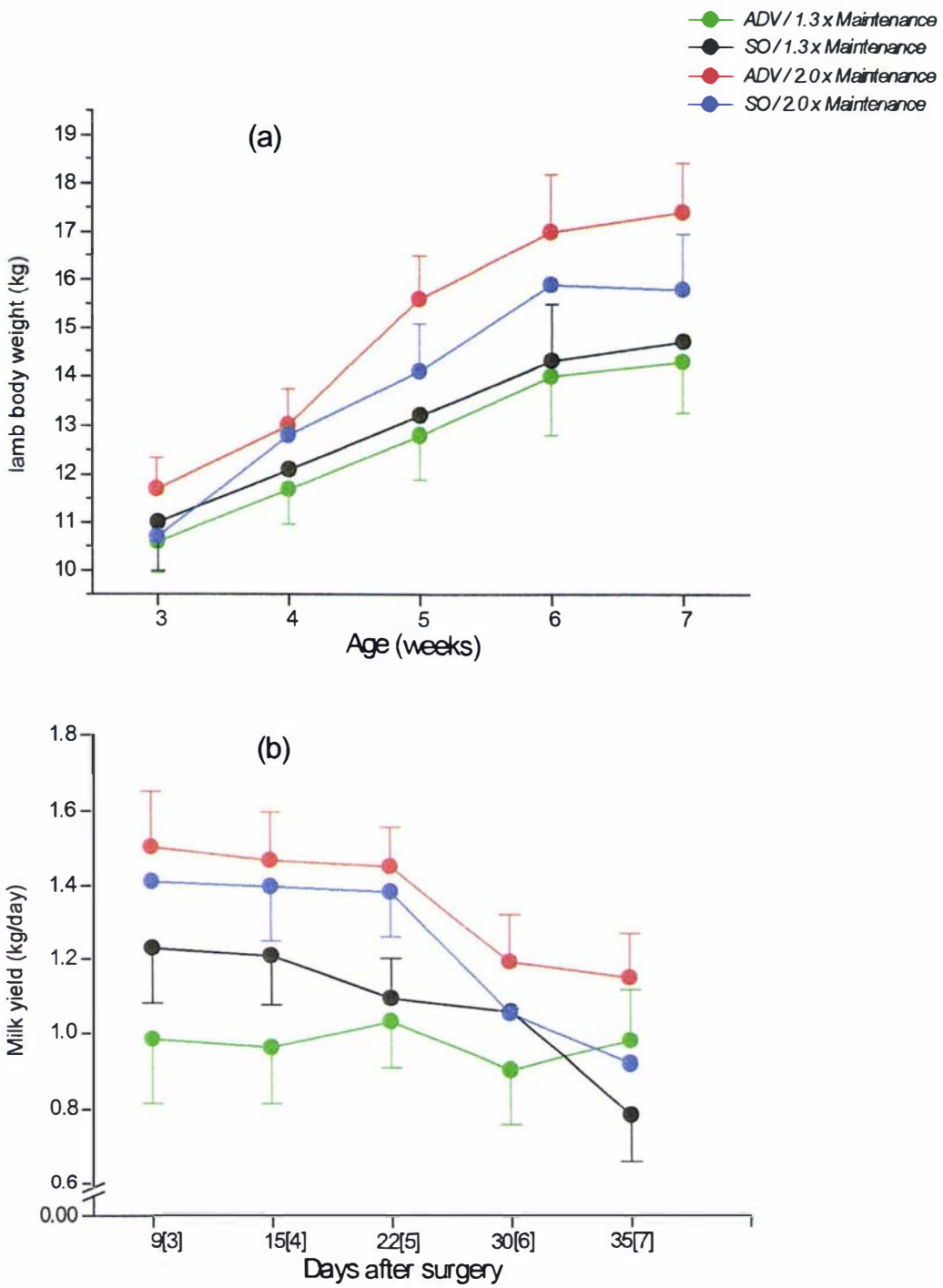

Figure 4.2 Body weight gains of lambs (a), and milk yield (b) in vagotomized (ADV) and sham-operated (SO) ewes fed at 1.3 x maintenance and 2.0 x maintenance intake. Legends are shown at top right hand corner. Within brackets on the 'x' axis of panel (b) are shown lactation weeks. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

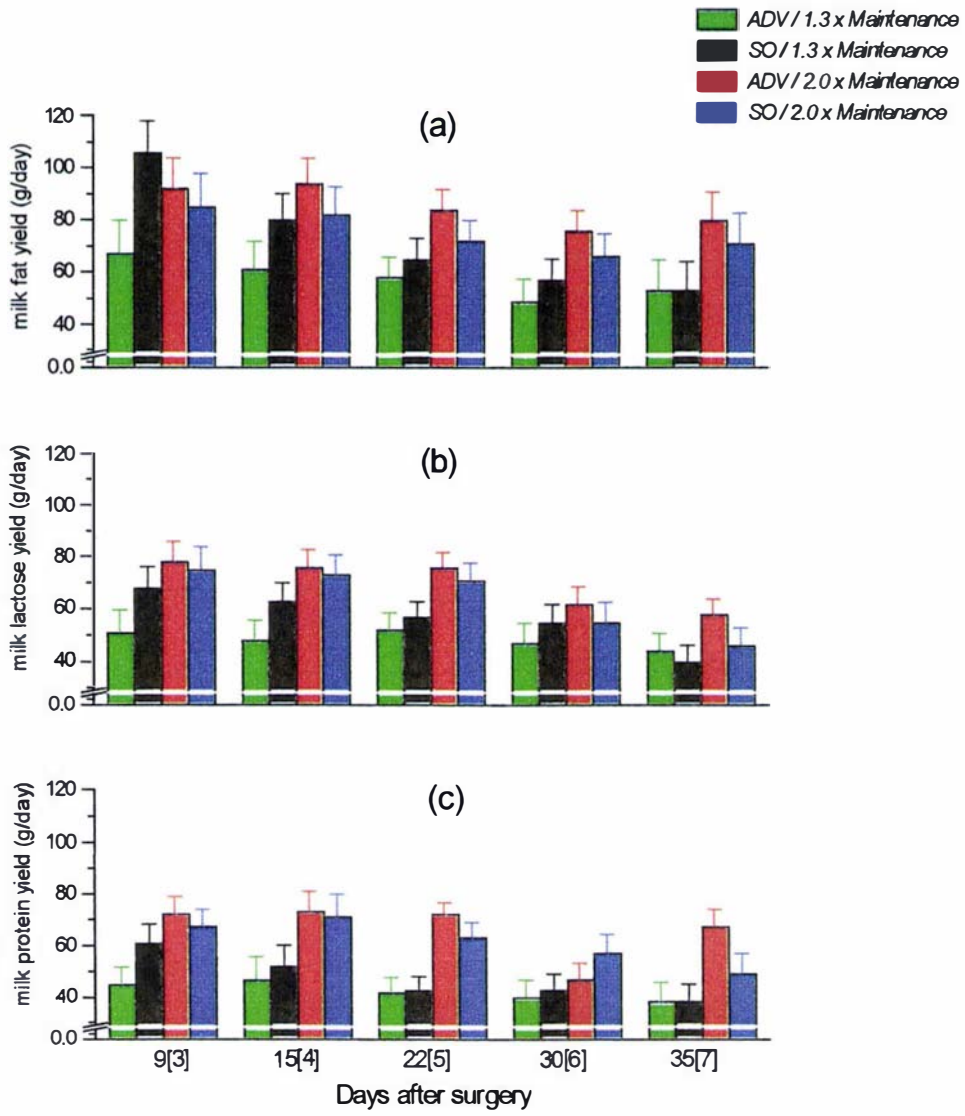

Figure 4.3 Milk fat (a), lactose (b) and protein (c) yields in lactating vagotomized (ADV) and sham-operated (SO) ewes fed at 1.3 x maintenance and 2.0 x maintenance intake. Legends are shown at top right hand corner. Within brackets on the 'x' axis of panel (b) are shown lactation weeks. Vertical bars: SEM.

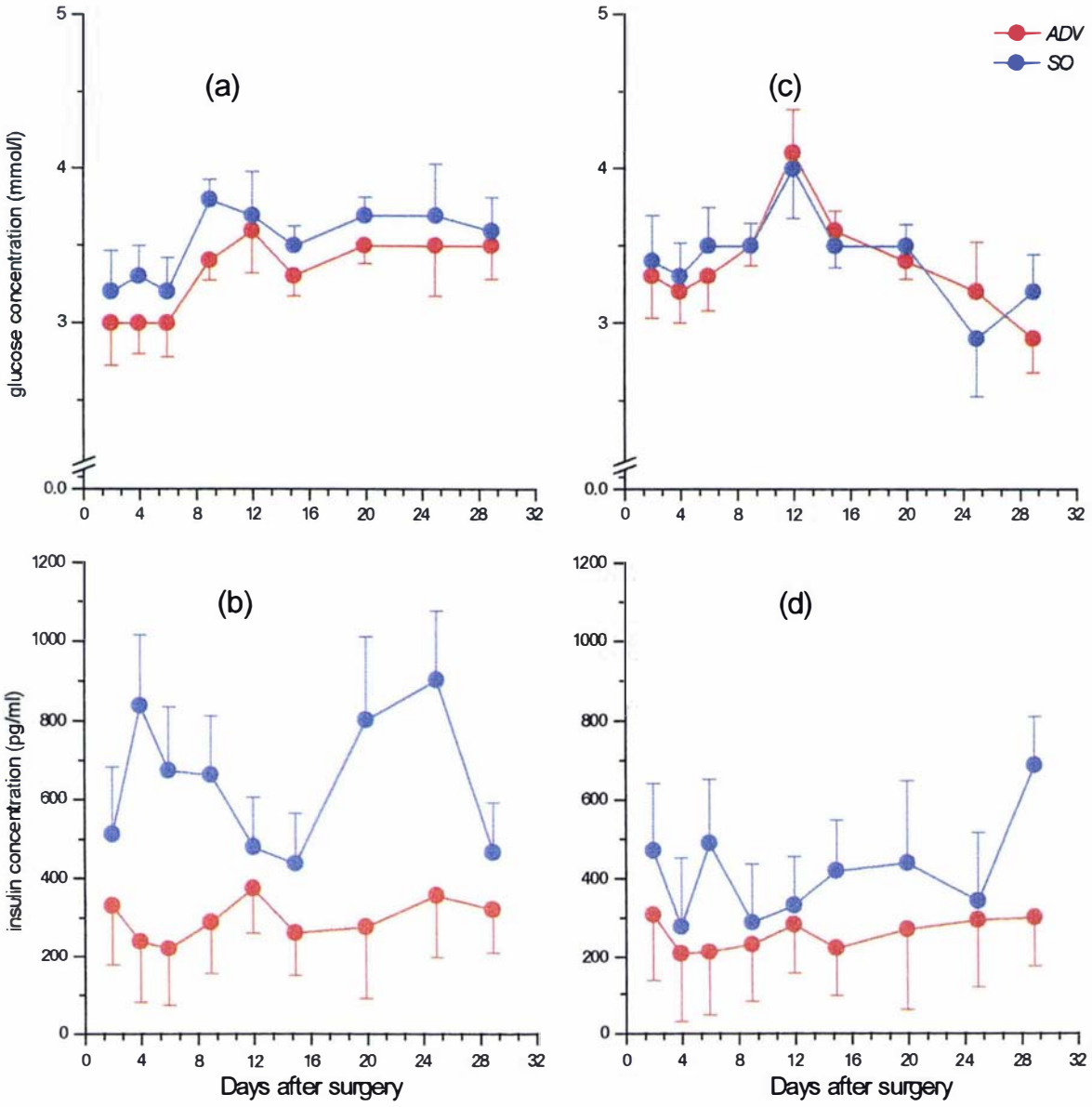

Figure 4.4 Baseline plasma concentrations of glucose and insulin in lactating vagotomized (ADV) and sham-operated (SO) ewes during a 29 day sampling period. Plasma glucose (a) and insulin (b) in ewes fed at 1.3 x maintenance, and glucose (c) and insulin (d) in ewes fed at 2.0 x maintenance intake. Legends are shown at top right hand corner. Vertical bars: SEM.

4.4.4 Plasma hormones and metabolites

There were no significant effects of either vagotomy or plane of nutrition on baseline concentrations of glucose (Fig. 4.4 (a),(c)). Plasma insulin concentrations were significantly ($P < 0.05$) lower in the ADV than in the SO ewes (Fig. 4.4 (b),(d)). The plane of nutrition had no effect on plasma insulin concentrations.

There were no significant effects of either vagotomy or plane of nutrition on baseline concentrations of PRL (Fig. 4.5 (a),(b)), OT (Fig. 4.5 (c),(d)), gastrin (Fig. 4.6 (a),(b)) and β-hydroxybutyrate (Fig. 4.6 (c),(d)).

4.4.5 Suckling-associated oxytocin release

Lambs immediately started to suckle their mothers when they were brought into the pens. Ewes started to eat immediately after food was offered. The difference in OT concentrations was significant ($P < 0.01$) and the OT concentrations were lower in the ADV than in the SO ewes (Fig. 4.7). When corrected for baseline values, OT concentrations in response to suckling were not different between ADV and SO ewes (data not shown). OT concentrations were not affected by vagotomy during suckling with simultaneous feeding (data not shown).

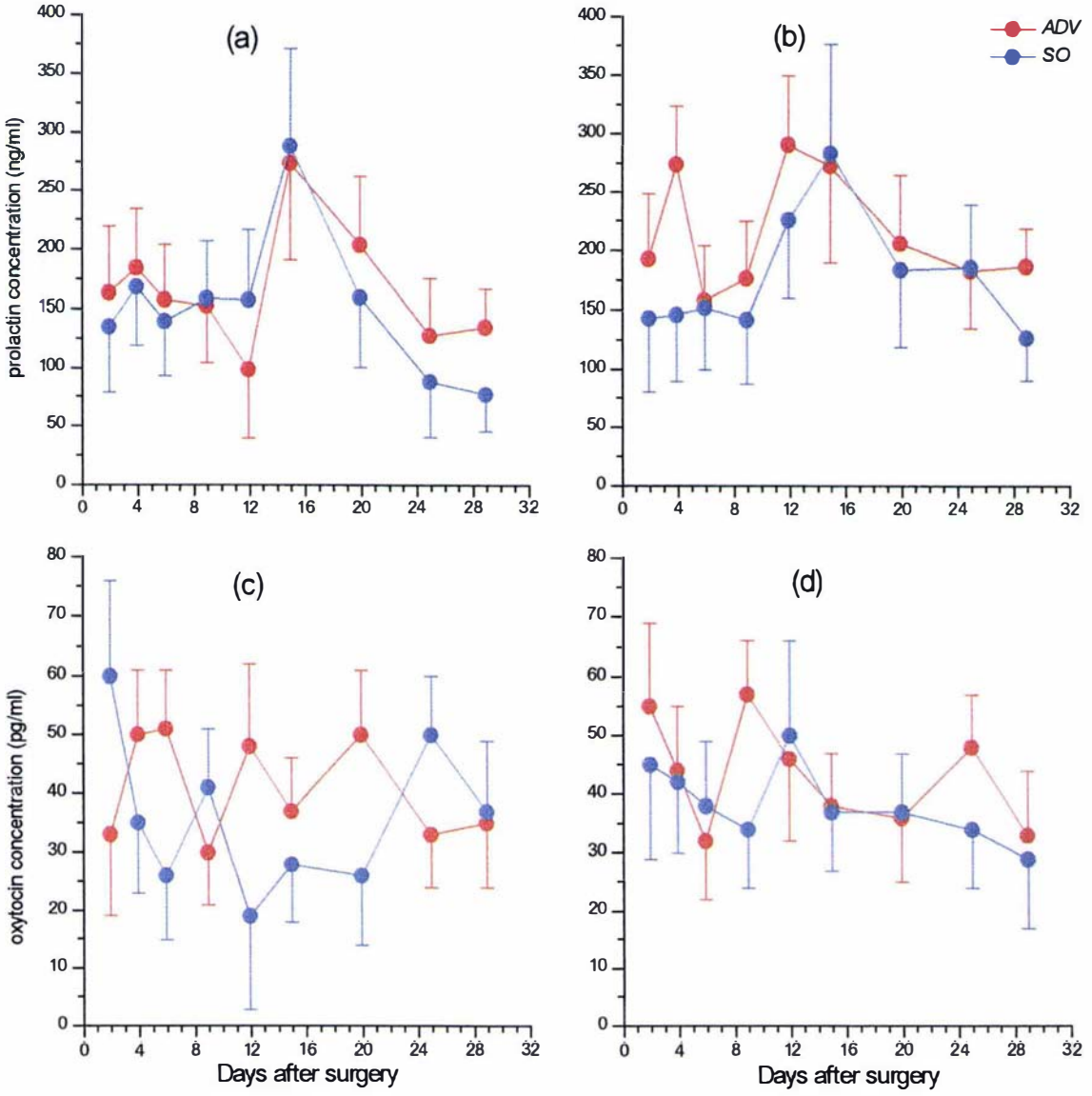

Figure 4.5 Baseline plasma concentrations of prolactin and oxytocin in lactating vagotomized (ADV) and sham-operated (SO) ewes during a 29 day sampling period. Plasma prolactin (a), oxytocin (c) in ewes fed at 1.3 x maintenance, and prolactin (b) and oxytocin (d) in ewes fed at 2.0 x maintenance intake. Legends are shown at top right hand corner. Vertical bars: SEM.

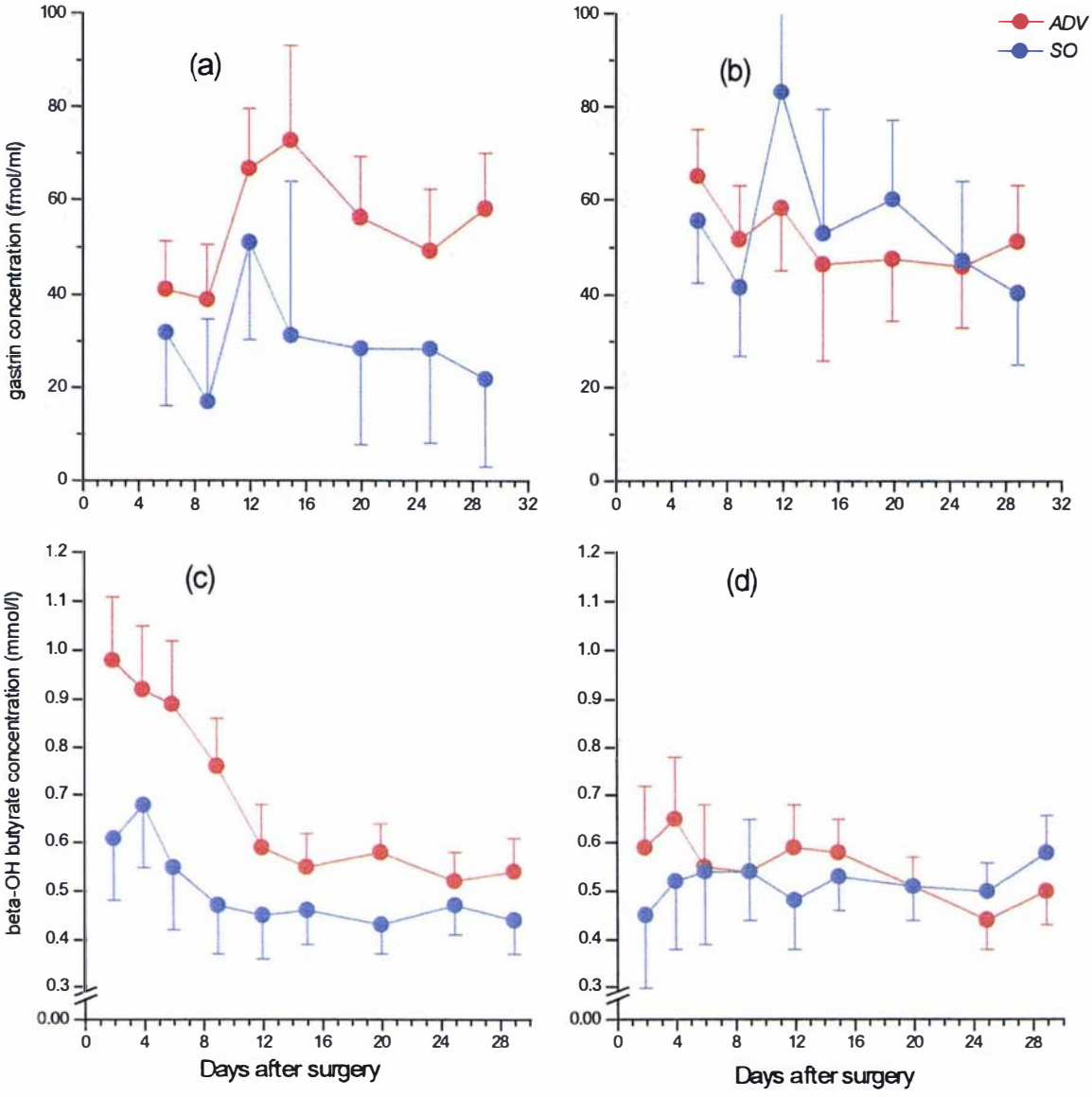

Figure 4.6 Baseline plasma concentrations of gastrin and β-hydroxybutyrate in lactating vagotomized (ADV) and sham-operated (SO) ewes during a 29-day sampling period. Plasma gastrin (a) and β-hydroxybutyrate (c) in ewes fed at 1.3 x maintenance, and gastrin (b) andβ-hydroxybutyrate (d) in ewes fed at 2.0 x maintenance intake. Legends are shown at top right hand corner. Vertical bars: SEM.

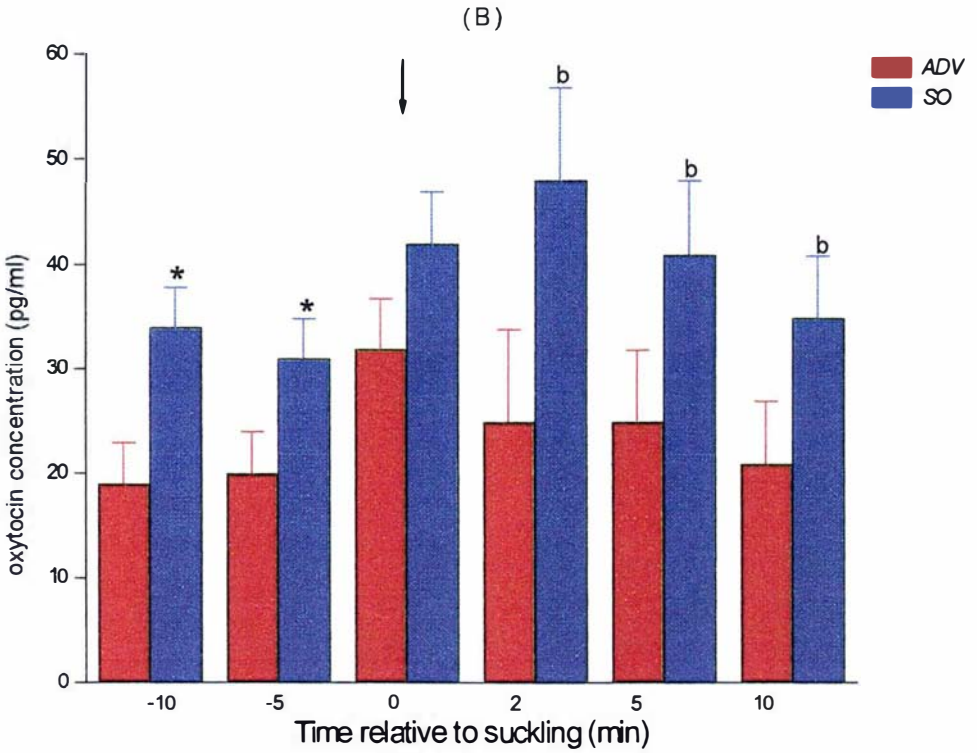

Figure 4.7 Plasma oxytocin concentrations before and during suckling in lactating vagotomized (ADV) and sham-operated (SO) ewes. Arrow indicates the time the lambs were returned to their dams after a 2-h separation period. Legends are shown at top right hand corner. $^{*}P < 0.05$, significantly different and, $^{b}P < 0.10$, tended to be different between treatment groups. Vertical bars: SEM.

4.4.6 Glucose challenge

4.4.6.1 Plasma Glucose

Following injection of glucose, no significant differences in glucose concentrations were found between ADV and SO ewes at either level of nutrition (Fig. 4.8 (a),(b) and Fig. 4.9 (a),(b)). Baseline corrected area under the glucose response curve was not different between ADV and SO groups (data not shown). However, glucose clearance rates from the blood tended to be lower ($P < 0.10$) in the ADV than in the SO ewes and the respective K values were 0.0605 ± 0.0066 and 0.0769 ± 0.0069.

4.4.6.2 Plasma insulin response

Plasma insulin concentrations following glucose injection were not affected by either plane of nutrition or vagotomy. Insulin concentrations tended to be lower ($P < 0.08$) in the ADV than in the SO group. Insulin concentrations following the first glucose challenge on PO day 12 were higher ($P < 0.01$) than that following the second glucose challenge on PO day 28 (Fig. 4.8 (c),(d) and Fig. 4.9 (c),(d). When baseline-corrected total area under the response curve for 30 min was measured, however, only day effect (i.e. day of glucose challenge) was significant ($P < 0.01$) (data not shown).

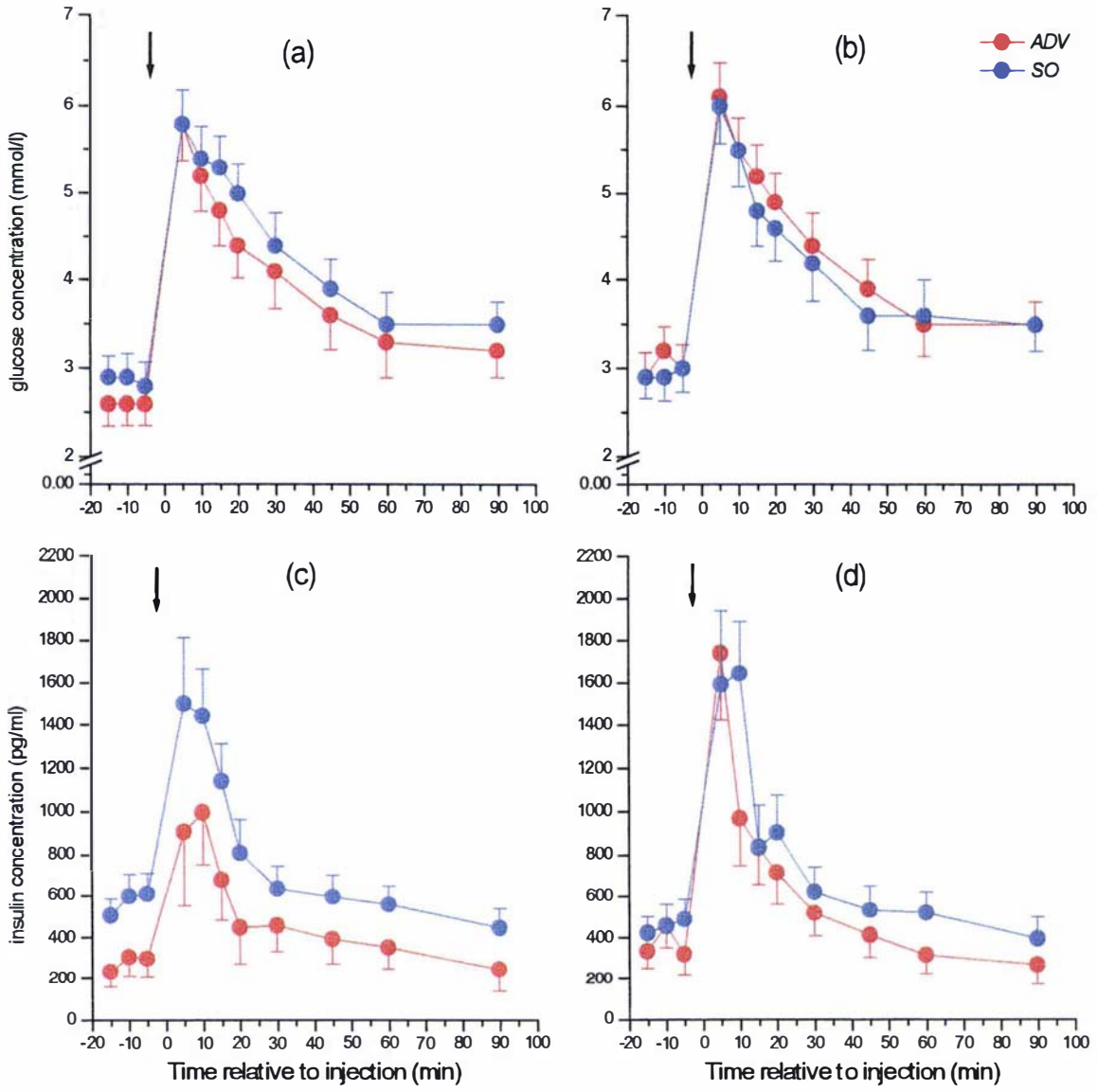

Figure 4.8 Plasma glucose and insulin concentrations in vagotomized (ADV) and sham operated (SO) ewes before and after intra-jugular glucose injection at day 12. Glucose (a) and insulin (c) in ewes fed at 1.3 x maintenance, and glucose (b) and insulin (d) in ewes fed at 2.0 x maintenance intake. Legends are shown at top right hand corner. Arrow indicates the time of glucose injection. Vertical bars: SEM.

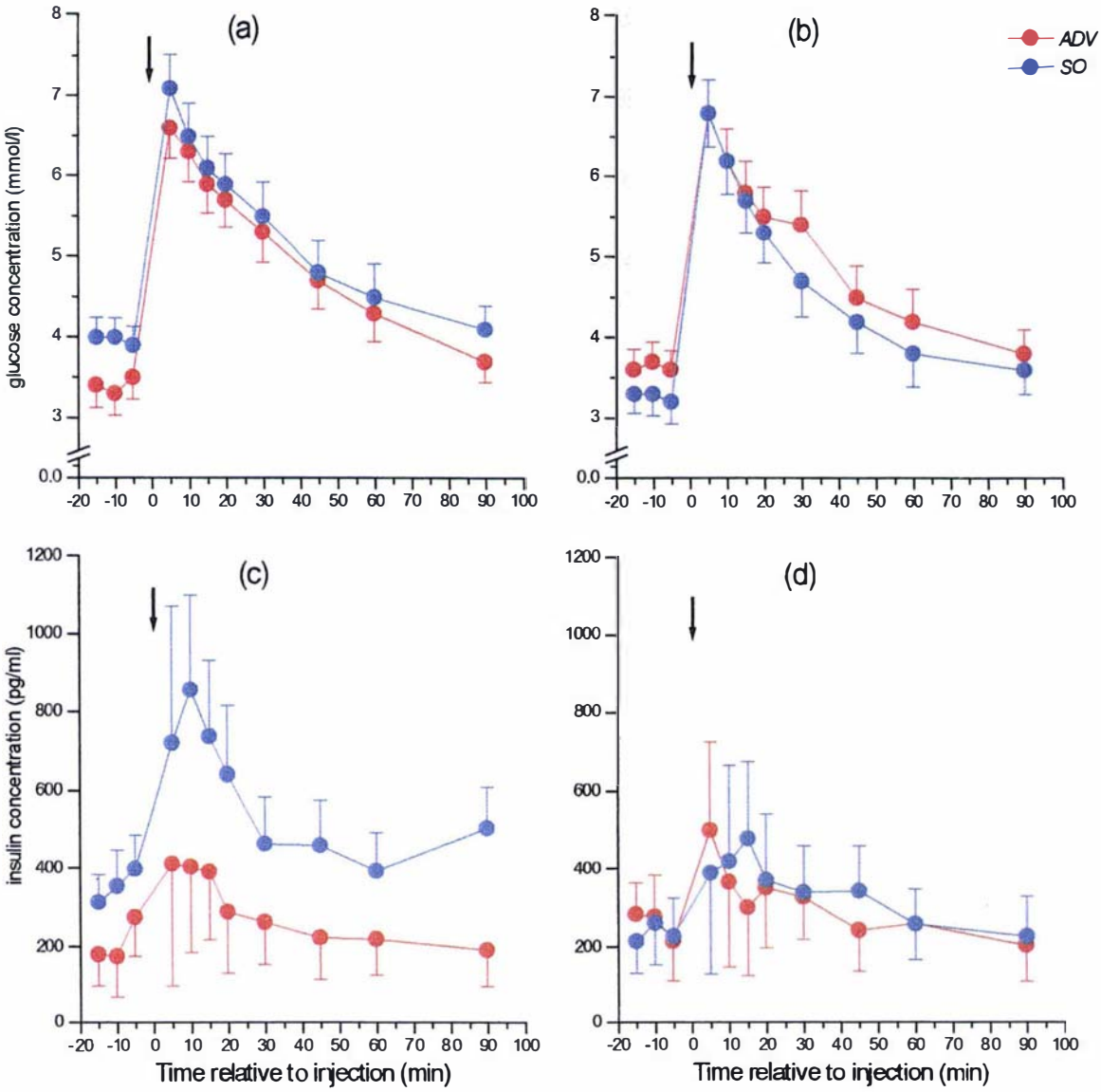

Figure 4.9 Plasma glucose and insulin concentrations in vagotomized (ADV) and sham-operated (SO) ewes before and after intra-jugular glucose injection at day 28. Glucose (a) and insulin (c) in ewes fed at 1.3 x maintenance, and glucose (b) and insulin (d) in ewes fed at 2.0 x maintenance intake. Legends are shown at top right hand corner. Arrow indicates the time of glucose injection. Vertical bars: SEM.

4.4.7 Physiological confirmation of vagotomy

Figure 4.10 shows the contractions of the reticulum, abomasum and duodenum and aortic blood pressure in a ADV and SO ewe in response to the electrical stimulation of the peripheral ends of the cut cervical vagus nerves. There were pronounced contractions of the reticulum in response to the stimulation in both SO ((a),(b)) and ADV ((c),(d)) ewes. Contractions of the abomasal body, although prominent in the SO ewe, were absent in the ADV ewe. There were sporadic increases in pressure in the duodenum during left cervical vagus nerve stimulation of the SO ewe (a) but it was absent in the ADV ewe (c). The stimulus had only minor effect on the heart as reflected in a small change of blood pressure during 10 min period ((a),(b),(c) and (d)).

Plasma insulin concentrations following vagal stimulation were log transformed to stabilize the variation before they were analysed in the repeated measures analysis. The overall effect due to vagotomy was significant ($P < 0.05$) and that insulin concentrations following stimulation were significantly ($P < 0.05$) lower in the ADV than in the SO ewes at all times except at 30 min where the concentration tended to be lower ($P < 0.06$) in the ADV than in the SO ewes (Fig. 4.11). There was no statistical indication of the laterality of the vagus nerves in stimulating insulin release although stimulation of the left cervical vagus nerve appeared to be a little more effective in causing insulin release than stimulation of the right cervical vagus nerve (data not shown).

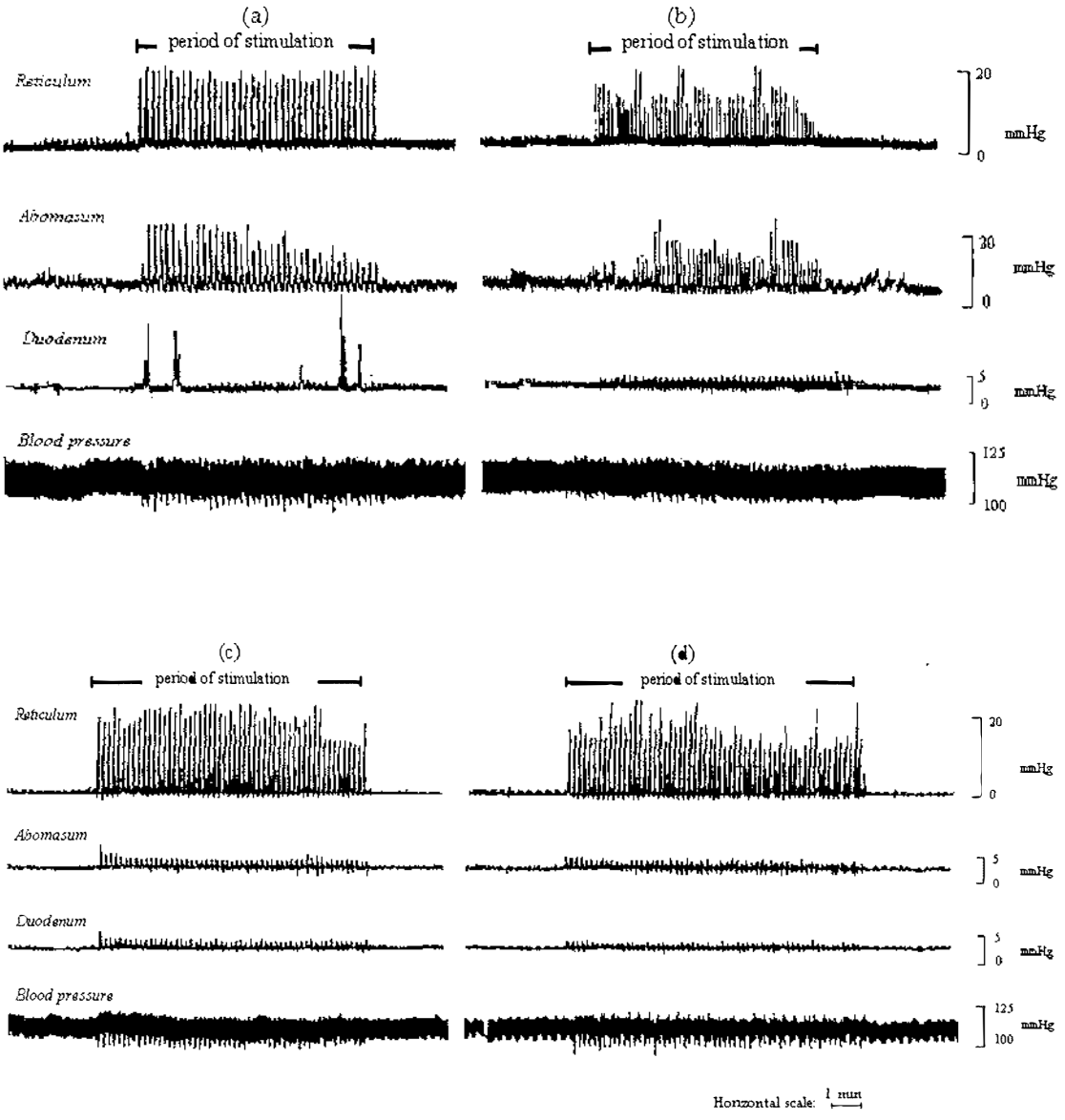

Figure 4.10 Physiological confirmation of vagotomy. Contractions of the reticulum, abomasum, duodenum and blood pressure recorded during electrical stimulation of the peripheral ends of the left cervical vagus (a) and right cervical vagus (b) nerves in a sham-operated ewe and the left cervical vagus (c) and right cervical vagus (d) nerves in a vagotomized ewe. Stimulus was delivered continuously for 10 min using short bursts of 1 sec duration every 10 sec (10 mA at 40 Hz). Chart speed was set at 0.1 mm/sec.

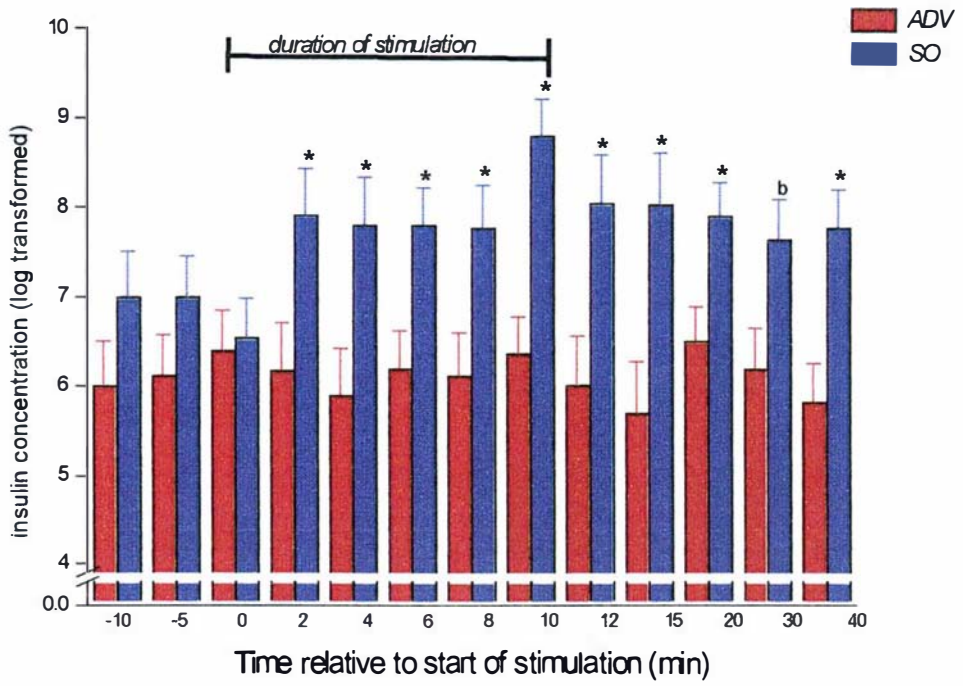

Figure 4.11 Plasma insulin response in lactating vagotomized (ADV) and sham-operated (SO) ewes in response to the electrical stimulation (using short bursts of 1 sec duration every 10 sec set at 10 mA and 40 Hz for 10 min) of the peripheral ends of the cut cervical vagus nerves in anaesthetized ewes. Legends are shown at top right hand corner. Plasma insulin concentrations were log transformed to stabilize the variation. $^{*}P < 0.05$, significantly different; $^{b}P < 0.06$, tended to be different, between treatment groups. Vertical bars: SEM.

4.5 DISCUSSION

The present study investigated the role of the vagal innervation of the abomasum, pylorus and upper small intestine (duodenum) on milk yield, baseline concentrations of hormones and metabolites, suckling-associated OT release and also insulin release in response to intravenous glucose injection and electrical stimulation of the cervical vagus nerves in lactating ewes.

The vagotomized ewes had reduced insulin concentrations at both planes of nutrition and the changes in insulin concentrations between sampling days were apparently much less than that seen in the sham-operated ewes (Figure 4.4 (b),(d)). This indicates a lack of vagal inputs to the pancreas from the GI tract and a role for the vagus nerve in the maintenance of baseline concentrations of insulin in lactating ewes. However, changes in plasma insulin concentrations in the sham-operated and vagotomized animals were not

reflected in the baseline concentrations of plasma glucose. Furthermore plasma glucose concentrations were not affected by either vagotomy or plane of nutrition. Relatively constant plasma glucose concentrations are most likely to be due to a tight glucose homeostasis that occurs in lactating ruminants. There were no exact relationships found between plasma glucose and insulin concentrations. Trenkle (1970) reported that changes in plasma glucose concentrations in fed and fasted sheep, even fasting for 48 to 72 h, were not large.

Insulin secretion in response to glucose injection was not affected by plane of nutrition. Insulin concentrations, however, tended to be lower in the vagotomized than in the sham-operated ewes. This trend was evident in the glucose clearance rate, which was lower in the vagotomized ewes compared with that of sham-operated ewes. The inability to demonstrate a significant effect of vagotomy on insulin release in response to glucose injection in lactating ewes was unexpected since the vagotomy suppressed insulin release in lactating ewes of experiment 1 (Chapter 2). However, it is possible that time of glucose injection relative to feeding is important to get any effect of vagotomy on insulin release as the presence of food in the GI tract may be crucial for sensitizing the pancreatic β-cells through vagal inputs. So that in experiment 2 (Chapter 3) and the present experiment, time of glucose injection relative to feeding may have influenced the results. In experiment 1, lactating vagotomized ewes were well fed and the glucose challenges were carried out 3-4 h after feeding (once a day feeding). In that experiment, insulin secretion in response to glucose injection was significantly suppressed in the vagotomized ewes. In experiment 2 (Chapter 3), wethers were fed hourly so that the amount of food ingested at each time was very small. In the present experiment, lactating ewes were fed twice a day and the glucose challenges were carried out 6-7 h after feeding. Lactating ewes in experiment 1 had the highest insulin response whereas animals in experiment 2 and the present experiment had comparatively small insulin responses but the responses in the present experiment were apparently larger than those of experiment 2. Bassett (1971) reported that basal insulin concentrations are positively correlated to the amount of digestible nutrient intake, suggesting that the presence of nutrients in the GI tract is important in sensitizing the pancreatic β-cells and that this sensitivity presumably occurs through the vagal signals carried from the GI tract to the pancreas. Under such situations, it is likely that if

intravenous glucose injection is given a short time after feeding then the insulin response the pancreatic β-cells elicit would be higher in a control animal whereas it would be lower in a vagotomized animal. Therefore, a further experiment was necessary in order to explore in detail the interaction between vagotomy and time of challenge relative to feeding on insulin release in response to intra-jugular injection of glucose.

Insulin response was, however, lower following the second glucose challenge compared to the first challenge. The reason for this apparent decreased insulin response approximately 2 weeks after the first glucose challenge is not clear, despite the hypothesis that the insulin responses are higher during subsequent glucose loads when the energy balance becomes higher. The ewes in both groups of intake levels were neither losing nor gaining body weight during the period when the second glucose challenge was undertaken (Figure 4.1 (b)), so that they probably had a better energy balance at this time compared to that during the first glucose challenge. But, at this stage of lactation milk yield had started to decline (Figure 4.2 (b)), particularly in ewes fed on the high plane of nutrition. An increased demand for milk synthesis created by intensely suckling lambs at this time of lactation, may have triggered mechanisms which may have caused a decrease in responsivity of pancreatic β-cells to both blood borne and neural signals. Thus, decreases in plasma insulin concentrations (Figure 4.9 (c),(d)) would favour increased circulating concentrations of nutrients that can be diverted to the mammary gland for milk synthesis. The increased suckling intensity may perhaps be reflected in an increasing body weight of the lambs during this period (Figure 4.2 (a)).

Plasma concentrations of β-hydroxybutyrate are a reflection of the balance between synthesis, either from rumen fermentation or by liver ketogenesis and peripheral tissue removal for use as an energy source in ruminants (Tauveron *et al.*, 1995) and it was felt necessary to monitor its concentration to see any effect of vagotomy on the partitioning of nutrients. Concentrations of β-hydroxybutyrate were generally higher in the vagotomized ewes than those of the sham-operated ewes fed on the low plane of nutrition but the overall difference was not statistically significant. No differences were detected in concentrations of plasma gastrin, PRL and OT between vagotomized and sham-operated ewes, probably because the concentrations were highly variable among ewes. The effect of

suckling may have contributed to the variation in baseline hormone concentrations since lambs were with the mothers at all times except during the days in which ewes were milked. It has been reported that suckling and/or milking stimulates the release of gastrin in pigs, dogs and cows (Uvnas-Moberg *et al.*, 1984; Linden *et al.*, 1987; Svennersten *et al.*, 1989; Samuelsson *et al.*, 1996a, 1996b), PRL in pigs, rats, cows and women (Tay *et al.*, 1996; Rojkittikhun *et al.*, 1993; Eriksson *et al.*, 1994; Samuelsson *et al.*, 1996a, 1996b), and OT in pigs, dogs, rats, sheep, cows and women (Yokoyama *et al.*, 1994; Rojkittikhun *et al.*, 1993; Eriksson *et al.*, 1987; Eriksson and Uvnas-Moberg, 1990; Eriksson *et al.*, 1994; Fuchs *et al.*, 1987; Svennersten *et al.*, 1995, 1992, 1990).

Oxytocin concentrations were significantly lower in the vagotomized ewes than in the sham-operated ewes (Figure 4.7), although the difference between two groups in the area under the OT response curve to suckling, corrected for baseline, was not significantly different. However, it is possible that the increased baseline concentration of OT in the sham-operated ewes before suckling began is a consequence of the influence of the exteroceptive stimuli emanated from the lambs. Fuchs *et al.* (1987) have demonstrated that the lactating ewes responded with release of OT in response to the sight, smell and/or sound which emanated from the lambs (see Figure 1.3). They further reported that this reflex release of OT in response to those exteroceptive stimuli became conditioned very rapidly. Oxytocin release during suckling with simultaneous feeding was not significantly different between two treatment groups. As discussed in Section 1.4.5.1, Fuchs *et al.* (1987) reported that suckling-associated OT release in lactating ewes was not further enhanced by feeding. In contrast, Svennersten *et al.* (1995) have demonstrated that feeding dairy cows during milking, enhanced milking-induced OT release and milk production whereas the cows deprived of food had low basal and milking-induced OT levels. Feeding-associated OT release has been reported in other species (Uvnas-Moberg *et al.*, 1985). Since the sampling in the present experiment was performed in three consecutive days, it is also possible that the ewes may have become conditioned to the sight and sound of the lambs and subsequently had elevated OT concentrations in the sham-operated animals before start of suckling. This type of OT release without involving tactile stimulation of the mammary sensory nerves has also been reported in cows and goats (Peeters *et al.*, 1960; Cleverley and Folley, 1970; McNeilly and Ducker, 1972;

Cowie, 1977). It is therefore possible that the difference in OT concentration in response to suckling was small due to the elevated baseline concentrations of the hormone in the sham-operated ewes. Alternatively, in the present experiment, peak OT in response to suckling may have gone undetected in sham-operated animals due to the sampling schedule relative to the pulsatile (brief period of secretion) nature of OT secretion, even under basal conditions in sheep (Mitchell *et al.*, 1982).

These results indicate that the release of OT under basal conditions and perhaps during suckling is moderated through the vagus nerves in lactating ewes. Eriksson *et al.* (1994) reported that suckling-associated OT release was significantly decreased in vagotomized rats compared to that of control rats. A decreased OT secretion after vagotomy is, therefore, a consequence of the interruption of the vagal network and thus the reduction in OT release resulting in a decreased efficiency of milk ejection could be one of the reasons that caused milk production to fall in vagotomized rats (Eriksson *et al.*, 1994; Uvnas-Moberg, 1989). Indeed the influence of vagal nerve activity on plasma levels of OT (Neumann *et al.*, 1994; Svennersten *et al.*, 1992; Stock and Uvnas-Moberg, 1988; Renaud *et al.*, 1987; Verbalis *et al.*, 1986; Smith *et al.*, 1985), together with relationships between OT producing nuclei and vagal nuclei in the hypothalamus (Siaud *et al.*, 1989; Sawchenko *et al.*, 1988; Sims and Lorden, 1986; Kooy *et al.*, 1984; Sawchenko and Swanson, 1982; Saper *et al.*, 1976), is well documented, which were discussed in Section 1.4.5.1.

The ewes fed on the high plane of nutrition produced more milk than those fed on the low plane of nutrition but there was no effect of vagotomy on the amount of milk or milk solids produced. In light of the lower OT secretion in the vagotomized animals, it is unclear why milk yield was not affected by vagotomy. One possible explanation is that because the milk volumes were measured by OT method, a real difference in the yield between the vagotomized and sham-operated animals, which would have otherwise been obtained, may have disappeared. Secondly, it is also possible that although OT release is essential for milk ejection reflex in many species including ruminants (Fuchs *et al.*, 1987; Tindal, 1978), a decrease OT concentration in lactating vagotomized ewes may have not been accompanied by a decrease in milk yield because of the anatomical architecture of the mammary gland in this species. In lactating ewes, like goats, an adequate milk removal can

still occur in the absence of OT because they have cisterns where milk excluded from alveoli can be stored (Tindal, 1978). In contrast, in rats, there are no sinuses or cisterns for the storage of milk so that a small change in OT concentration could alter the milk flow and removal (DeNuccio and Grosvenor, 1971). This is a possible explanation why milk yield was significantly decreased in lactating vagotomized rats (Eriksson *et al.*, 1994), but not in lactating vagotomized ewes in the present and previous study (Chapter 2).

Body weight gains of lambs are in line with milk yield data. Lambs that suckled HP mothers had higher body weights than those suckled LP mothers. However, body weight increases from 3 weeks to 6-7 weeks of age were higher ($P < 0.01$) in lambs that suckled SO-HP mothers than those suckled ($P < 0.05$) ADV-HP mothers. This indicates that lambs of SO-HP mothers were getting more milk than the others.

Electrical stimulation of the peripheral ends of the cut cervical vagus nerves did not elicit contractions of the abomasum and duodenum in vagotomized ewes, demonstrating that vagotomies performed were complete (Figure 4.10). The completeness of the vagotomy was further confirmed by the findings that insulin release in response to electrical stimulation of the cervical vagus nerves was significantly suppressed in vagotomized ewes compared to that of sham-operated control ewes (Figure 4.11). This confirms the results of Pierzynowski *et al.* (1986) who demonstrated that electrical stimulation of the vagus nerve caused insulin secretion in sheep. Furthermore, these data that indicate an effect of vagotomy on insulin secretion in response to vagal stimulation are strongly supported by findings from the literature (Adrian *et al.*, 1983; Bloom and Edwards, 1981; Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Holst *et al.*, 1981; Daniel and Henderson, 1967). These authors have shown that pre-treatment of calves with atropine effectively suppressed the rise in plasma insulin concentration in response to vagal stimulation (Adrian *et al.*, 1983). Furthermore, insulin secretion in response to electrical stimulation of either the ventral or the dorsal vagi was significantly reduced in rats in which either the gastric or hepatic branches were cut whereas insulin release was not affected in animals which underwent coeliac branch vagotomy (Berthoud and Powley, 1990; Berthoud *et al.*, 1990). These results, together with findings of the present and previous experiments (Ami *et al.*, 1993; Kirchgessner and Gershon, 1990, 1989; Laughton and Powley, 1987; Poulsen *et al.*, 1983; Matsuo and Seki, 1978; Stavney *et al.*, 1963),

which were discussed in Section 1.4.3.1, suggest that the vagus nerves play an important role in the release of insulin from the pancreas in a variety of species including lactating sheep. The present results further confirm the previous findings (Chapter 2) that pancreatic β-cells are innervated either directly or indirectly (Kirchgessner and Gershon, 1990, 1989) by vagal efferent fibres carried in the abomasal, pyloric and duodenal branches of dorsal and ventral vagus nerves that innervate the abomasum, pylorus and duodenum in sheep.

In conclusion, the results do not completely support the idea that suppression of insulin release in response to glucose injection is a general effect of the vagotomy in lactating ewes. The difference in insulin responses between experiments (i.e. Chapter 2 to Chapter 4) may be closely associated with the pattern of feeding and time of glucose injection relative to feeding. This needs to be further elucidated in a experiment in which the glucose challenge is carried out at more appropriate times, eg. a short time after feeding and during fasting conditions. It is important to know whether the magnitude of plasma insulin response during hyperglycaemia is dependent on the degree of β-cell sensitivity caused by the presence of food through the vagal inputs from the GI tract. This will help clarify whether or not insulin response to glucose injection is a function of an activated vagal neurons in the presence of food in the GI tract. However, the results indicate that the vagus nerve plays a role in the regulation of baseline insulin concentrations although its role in the regulation of plasma concentrations of gastrin and PRL is not clear. Results further indicate that the release of OT under basal conditions, and perhaps in response to suckling, is mediated through the vagus nerve in lactating ewes. Further, it is possible that in the present experiment, peak OT in response to suckling may have gone undetected in sham-operated animals due to the sampling schedule relative to the brief period of OT secretion. Therefore, this needs to be further investigated with samples taken at more frequent intervals during suckling. Milk yield was not affected by vagotomy, possibly because of either exogenous OT injected before each milking or the difference in the anatomical architecture of the mammary gland from that of the rat. Alternatively there may be no dose-response relationship between OT and milk yield.

CHAPTER 5

THE VAGAL CONTROL OF INSULIN RELEASE IN FED AND FASTED WETHERS

5.1 ABSTRACT

The role of the vagal innervation of the abomasum, upper small intestine and liver in insulin release was studied in wethers. Measurements were made of the insulin secreted in response to glucose injection, electrical stimulation of the cervical vagus nerves and during the postprandial and cephalic phase responses to feeding. Surgical preparations consisted of abomasal and duodenal vagotomy (ADV; n=8), hepatic, abomasal, and duodenal vagotomy (HADV; n=10) and sham-operations (SO; n=8). After surgery the wethers were randomly assigned to 2 feeding groups; 13 wethers (ADV=4; HADV=5; SO=4) on a low plane of nutrition (LP) and 13 wethers (ADV=4; HADV=5; SO=4) on a high plane of nutrition (HP). Feed requirements at LP (0.8 x maintenance) and HP intake levels (1.4 x maintenance) were calculated from individual body weights. Food was offered once a day at 1100 h. On PO days 10 and 13, all wethers were given an intravenous injection of glucose (100 mg/kg bw), either 2 h (2h-PF) or 22 h (22h-PF) after feeding and jugular blood samples were collected for glucose and insulin determinations. Intake levels were then reversed and the glucose injections repeated on PO days 19 and 22. On PO days 9 and 18, jugular blood samples were collected at -3, -2, -1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 h relative to time of feeding to study the postprandial concentrations of plasma insulin and glucose. On PO day 28, CPIR was investigated in ADV (n=8), HADV (n=10) and SO (n=8) wethers. A terminal acute experiment was undertaken in randomly selected ADV (n=4), HADV (n=4) and SO (n=4) wethers to study the effect of vagotomies on insulin release in response to an electrical stimulation of the peripheral ends of the cut cervical vagus nerves. Glucose injection caused plasma glucose to rise to significantly ($P < 0.01$) higher levels in all treatment groups. Glucose removal rate was not significantly different between treatment groups. Plasma insulin secretion following glucose injection was significantly ($P < 0.05$) suppressed in the ADV and HADV groups and the baseline corrected total area under the response curve was significantly ($P < 0.05$) lower in the ADV and HADV groups than in the SO group. Insulin secretion in response to glucose injection 2h-PF was significantly ($P < 0.0002$) higher than that after glucose injection at 22h-PF. Postprandial concentrations of plasma glucose was not significantly different between treatment groups. Plasma insulin response was highly significantly ($P < 0.0002$) suppressed in the ADV and HADV groups in comparison with the SO group and,

it was significantly ($P < 0.05$) higher in the wethers of HP than in those of LP group. CPIR was significantly ($P < 0.05$) suppressed in the ADV and HADV groups compared with that of the SO group after the start of ingestion of food. Total insulin response area, corrected for baseline, was significantly ($P < 0.05$) lower in the ADV and HADV groups than in the SO group but the glucose concentrations were not different between treatment groups. There were no differences detected between the 2 levels of vagotomy i.e. ADV and HADV. There was an insulin response to electrical stimulation of the vagus in the SO animal of the first 3 animals one from each of the 3 treatment groups. These results indicate that the vagal innervation of the GI tract modulates pancreatic insulin secretion in response to intra-jugular injection of glucose in wethers. The results support the concept that the suppression of insulin release in response to glucose injection is a general response to cutting the vagal innervation to the GI tract and the effect is not confined to lactating ewes but also occurs in wethers. The results provide further support for the hypothesis that the magnitude of the plasma insulin response during hyperglycaemia is dependent on the degree to which the β-cells have been sensitised by vagal neurons activated by the presence of food in the GI tract. The results further indicate that insulin release in response to feeding (postprandial), feeding-associated rapid cephalic phase and electrical stimulation of the cervical vagus nerves is also modulated by vagal neurons contained in the abomasal, pyloric and duodenal branches of the vagus nerves in wethers.

5.2 INTRODUCTION

The release of pancreatic insulin in response to the intra-jugular injection of glucose was suppressed by selective vagotomy of the abomasum, pylorus and duodenum in lactating ewes (Chapter 2). Based on these results it was suggested that the depressed insulin release during hyperglycaemia in the vagotomized ewes was a more general response to cutting the vagal innervation of the GI tract. This led to the study of the effect of vagotomy in greater detail in wethers because they were considered to be a more convenient model (Chapter 3). However, results from that experiment were not conclusive, possibly because of interactions with the pattern of feeding used. Alternatively, it was suggested that the vagal innervation of the GI tract may modify pancreatic insulin release during hyperglycaemia and that this response is confined to lactating ewes.

Therefore, a further experiment (Chapter 4) was undertaken once more in lactating ewes to explore in detail the effect of vagotomy on insulin release and also the effects of different planes of nutrition on the insulin response. However, the results did not support the idea that the effect of vagotomy on insulin release may be confined to lactating ewes. Rather the results indicated that insulin release in response to glucose injection in vagotomized animals may be dependent on the time of glucose injection in relation to time of feeding.

In experiment 1 (Chapter 2), lactating vagotomized ewes were fed *ad libitum* and the glucose challenges were carried out 3-4 h after feeding (once a day feeding). In experiment 2 (Chapter 3), wethers were fed hourly so that the amount of food ingested at each time was very small. In experiment 3 (Chapter 4), lactating ewes were fed twice a day and the glucose challenges were carried out 6-7 h after feeding. Lactating ewes in experiment 1 had the highest insulin response whereas animals in experiments 2 and 3 had comparatively small insulin responses but the responses in experiment 3 were apparently larger than those of experiment 2 (Section 4.5 of Chapter 4). A strong positive correlation between basal insulin concentrations and digestible nutrient intake has been documented (Bassett, 1971). This, together with the results from the three previous experiments, indicate that pancreatic insulin secretion is amplified if an intravenous glucose load is administered when the pancreatic β-cells are highly sensitized due to the presence of food in the GI tract. Under such situations, the vagus nerve may play a dominant role in transmitting signals from the GI tract to the pancreas. Therefore, the present experiment in wethers was undertaken to explore in detail the interaction between vagotomy and time of challenge on insulin release in response to intra-jugular injection of glucose.

It has also been reported that pancreatic insulin secretion in response to a variety of stimuli is suppressed following hepatic branch vagotomy in rats and guinea pigs (Nagase *et al.*, 1993; Lee and Miller, 1985; Niijima, 1982, 1983, 1986; Yamazaki and Sakaguchi, 1989). Therefore, it was of interest to study, in addition to the effect of abomasal/duodenal vagotomy, the effect of cutting the vagal innervation to the liver on insulin secretion in response to intra-jugular glucose injection in wethers. This will further provide evidence on the relative importance of the hepatic branch of the vagus nerve in the regulation of blood glucose concentrations in ruminants.

The sensory aspects of food before, and during, ingestion trigger neural reflexes that result in various motor and secretory responses in the GI tract and its accessory organs such as the pancreas. These food-related sensory stimuli, which include sight, smell, sound or taste of palatable food, have always been considered specific and effective elicitors of CPIR, i.e. the insulin released during a meal, but prior to absorption of nutrients from the GI tract. Although the functional significance of the cephalic phase reflexes has not been identified, it has been proposed that the importance of this type of responses is to prepare the viscera for an efficient digestion and utilization of post-absorptive nutrients (Strubbe and Steffens, 1975).

It was suggested that the cephalic phase reflexes including CPIR are a group of physiological responses that occur after activation of the vagus nerve through food-related sensory stimulation (Strubbe and Steffens, 1975; Berthoud *et al.*, 1981; Taylor and Feldman, 1982 and Section 1.4.4). Prior i.v. administration of atropine in rats (Strubbe, 1992; Berthoud and Jeanrenaud, 1982) and lambs (Porter and Bassett, 1979) completely suppressed meal-associated CPIR, suggesting that the vagus nerve is involved in the meal-associated CPIR in these species. Thus, it appears that sensory stimulation carried to the vagal centres in the brain stem initiates a rapid preabsorptive release of pancreatic insulin through activation of vagal efferent fibres to the pancreatic β-cells (Niijima, 1991; Niijima *et al.*, 1990). However, the use of systemically administered muscarinic blockers does not shed light on the neural pathways involved in ruminants.

The experiments described in this chapter were designed to demonstrate the effects of cutting the vagal innervation to the abomasum, pylorus, duodenum and liver in the release of pancreatic insulin in response to glucose injection, electrical stimulation of the cervical vagus nerves, feeding and cephalic phase food ingestion in wethers.

5.3 MATERIALS AND METHODS

5.3.1 Animals, housing and acclimatisation

Thirty Romney wethers, approximately one year old, were obtained from Aorangi farm of AgResearch Grasslands, Palmerston North. They were housed individually in

metabolism crates in covered yards at AgResearch for approximately 20 days prior to surgery.

Wethers were accustomed to housing, handling, feeding and other routine procedures and given water *ad libitum* at all times. They were fed lucerne pellets (12 MJ ME/kg DM) *ad libitum* for the first 10 days and then at 1.2 x daily maintenance intake level until surgery. They were also fed a small amount (less than 100 g/day) of lucerne chaff over the 20 day acclimatisation period. Food was offered once a day at 1100 h. Twenty six of those that adapted and were eating well were selected for the experiment. All animals underwent surgery and were randomly allocated to 3 treatment groups (for detail see Section 5.3.3). The mean body weight (± SE) at the time of start of the experiment was 31.8 ± 1.1 kg. All procedures involving wether sheep were approved by the Massey University and AgResearch Animal Ethics Committee.

5.3.2 Surgical procedures

Surgery was carried out as described in Section 2.3.2.1 (see also No. 1, 2 & 3 of Figure 1.1). In the present experiment, the hepatic branch of the ventral vagus nerve (see No. 4 of Figure 1.1) and the abomasal and pyloric branches of the dorsal vagus nerve (see No. 5 & 6 of Figure 1.2) were also sectioned. Jugular catheters were inserted as described in Section 2.3.2.2.

5.3.3 Experimental design

Allocation of wethers into different treatment groups in a cross-over experiment was carried out as follows. Surgery was carried out over four days. On each of the first 3 days, 6 randomly selected wethers underwent abomasal and duodenal vagotomy (ADV; n=2) or hepatic, abomasal and duodenal vagotomy (HADV; n=2) or sham-operation (SO; n=2), while on day 4 the remainder underwent surgery (ADV, n=2; HADV, n=4, and SO, n=2). Until the fourth day after surgery wethers continued to receive feed at 1.2 x daily maintenance intake level. They were then allocated to one of two planes of nutrition, 0.8 x daily maintenance (LP) or 1.4 x daily maintenance (HP). Daily allowances were calculated according to body weight (Rattray, 1986). Each treatment group, ADV-HP, ADV-LP, SO-HP and SO-LP, comprised 4 animals, 1 from each of the 4 days of surgery. Each of

the two HADV (HADV-HP and HADV-LP) groups comprised 5 animals, 1 from each of the first 3 days of surgery and 2 from the fourth day. Following the first set of glucose challenges (days 10 and/to 13 post-operation) the feeding levels were reversed; wethers on HP were switched to LP and *vice versa.*

5.3.3.1 Glucose challenge

Glucose challenges were given in two sets of two challenges, on PO days 10 and day 13 (S-1) and on PO days 19 and day 22 (S-2). On PO day 10, the first challenge of the S-1 was carried out 2 h post-feeding (2h-PF) in wethers that underwent surgery on days 1 and day 3. On PO day 13, they were challenged again, this time 22 h post-feeding (22h-PF). The procedure was repeated in the wethers that underwent surgery on day 2 and day 4, except that the first and second challenges of the S-1 were carried out 22h-PF and 2h-PF, respectively. The objective of this procedure was to eliminate any confounding effects of the time of feeding with time after surgery.

On PO day 14 the intakes of the wethers were crossed-over. After 5 days the second set of glucose challenges were given using the same protocol as that used for the S-1 so that the first and second challenge of the second set of challenges were carried out on PO days 19 and day 22, respectively.

For each glucose challenge, wethers were given a bolus injection of glucose (100 mg/kg bw) through a jugular catheter inserted 2-3 days before the challenge. Sterile 40% (w/v) glucose solution (Baxter Healthcare Pty Ltd, NSW, Australia) warmed to body temperature was used for intra-jugular injections. Blood samples were collected at -15, -10, -5, 5, 10, 15, 20, 30, 45, 60, and 90 min relative to glucose injection from a contralateral jugular catheter.

5.3.3.2 Postprandial pattern of plasma glucose and insulin

Postprandial patterns of plasma glucose and insulin were investigated in all treatment groups. On PO day 9 and day 18, blood samples were collected at -3, -2, -1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9 h relative to feeding time.

5.3.3.3 Cephalic phase insulin release (CPIR)

This experiment was carried out on PO day 28. Blood samples were collected at -10, -5, -2, 0, 1, 2, 4, 6, 8, and 10 min relative to feeding time. Food was weighed a day before the experiment and kept in plastic bags out of the sight of the wethers until the time of feeding. On the day of the experiment blood samples were taken 10, 5 and 2 min before food was offered. Food was then offered suddenly but quietly and a blood sample taken immediately (i.e. time 0), and at 1, 2, 4, 6, 8, and 10 min after feeding began.

5.3.3.4 Physiological confirmation of vagotomy

This was carried out as a terminal acute experiment to physiologically confirm the vagotomies and also to study insulin release in response to electrical stimulation of the cervical vagus nerves. The experiment was carried out on 12 (ADV=4, HADV=4, and SO=4) randomly selected wethers. Preparatory surgery was carried out as described in Section 4.3.3.6. Right and left cervical vagus nerves were cut and the peripheral ends stimulated using a standard physiological stimulus (short bursts of 1 sec duration every 10 sec at 10 mA and 40 Hz), which was applied continuously for a period of 10 min. Blood samples were collected at -10, -5, 0, 2, 4, 6, 8, 10, 12, 15, 20, 30, 45, and 60 min relative to start of stimulation. Harvested plasma was analysed for insulin only.

5.3.4 Blood processing and sample analyses

All blood samples (5 ml) were collected from a jugular catheter into 0.05 ml of 15% Na_2 EDTA (1.5 mg per ml blood) to prevent clotting. The samples were stored in ice and centrifuged at 4000 rpm for 15 min at 4° C immediately after completing the experiment. The resulting plasma was stored at -20^0 C, until analysed for plasma metabolites and hormones. Plasma concentrations of glucose and insulin were determined as described previously (Section 2.3.4).

5.3.5 Statistics

Statistical significance between the means of treatment groups was determined using ANOVA designed to account for repeated measures. Duncan's multiple comparison range test was used to compare the means within individual treatment groups when the time and the interaction between time and treatment effects within subjects were

significant. During the glucose challenges, baseline corrected area under the response curve was determined for 90 min for glucose and insulin after the glucose injection, and for CPIR, baseline corrected area under the response curve was determined for 10 min after presentation of food. For glucose challenges, baseline corrected values for glucose at 5 to 30 min post-injection, were used to fit a linear regression model and the resulting values for glucose clearance constant (K) were used in the ANOVA to determine the significance of differences between glucose clearance rates. Data are presented as least square means ± standard errors (±SEM). A probability value less than 0.05 was considered significant. Statistical analyses were carried out using the computer package 'SAS' (The SAS System for Windows, Version 6.11, SAS Institute Inc., Cary, NC, U.S.A.).

5.4 RESULTS

5.4.1 General effects of vagotomy

It took 24-48 h following surgery for all wethers to return to pre-surgical levels of food intake. Body temperature was normal before and after surgery.

5.4.2 Food intake

During the experiments most wethers fed at 0.8 x maintenance intake level generally ate all food within 1 h and always within the first 2 h after food was presented. However, two of the wethers took up to 4 h to eat their food. About half of the wethers in each treatment group fed at 1.4 x maintenance intake level ate all of their food within the first 2 h. The remainder ate most of their food within the first 2 h of feeding and all of it within 6-7 h.

5.4.3 Glucose challenge

5.4.3.1 Plasma glucose

Glucose injection caused plasma glucose concentrations to rise significantly ($P < 0.01$) to about 7.5 mmol/l in all treatment groups at 2h-PF and 22h-PF at both the LP and HP levels (Fig. 5.1). Plasma glucose clearance rates, baseline corrected for 30 min, between treatment groups were not significantly different (data not shown). Baseline corrected total area under the curve to 90 min was significantly ($P < 0.01$) higher in

wethers fed on the low plane of nutrition compared to those fed on the high plane of nutrition (data not shown). It was also significantly ($P < 0.05$) higher in the wethers that received the glucose load 22h-PF than those that received the glucose load 2h-PF (data not shown).

5.4.3.2 Plasma insulin response

Analysis of plasma insulin data for main effects (between subject effects) using repeated measures design revealed that insulin concentrations following glucose injection were significantly ($P < 0.05$) greater in the SO group than in the ADV and HADV groups (Fig. 5.2). Following glucose injection, plasma insulin concentrations were significantly ($P < 0.0002$) higher when administered 2h-PF than at 22h-PF. Insulin response was significantly ($P < 0.001$) higher during S-2 when compared to that during S-1 (data not shown). None of the interaction effects were significant, however, the interaction effect between the plane of nutrition and vagotomy tended ($P < 0.10$) to be significant. ANOVA for within subject effects also showed that insulin concentrations were significantly ($P < 0.0002$) varied with time but were dependent on the vagotomy ($P < 0.05$), plane of nutrition ($P < 0.0002$), time of glucose injection relative to time of feeding ($P < 0.0002$) and time after surgery ($P < 0.0002$). Baseline corrected total area under the response curve to 90 min was significantly ($P < 0.05$) higher in the SO wethers than in the ADV and HADV wethers (Fig. 5.3 (a)). It was also found that the areas under the insulin response curve immediately after glucose injection, i.e. 5-10 min and 10-15 min, were significantly ($P < 0.05$) higher in the SO group than in the ADV group and HADV group (Fig. 5.3 (b)). Baseline corrected total area under the insulin response curve was significantly ($P < 0.01$) higher following challenges at 2h-PF than at 22h-PF (Fig. 5.3 (a)).

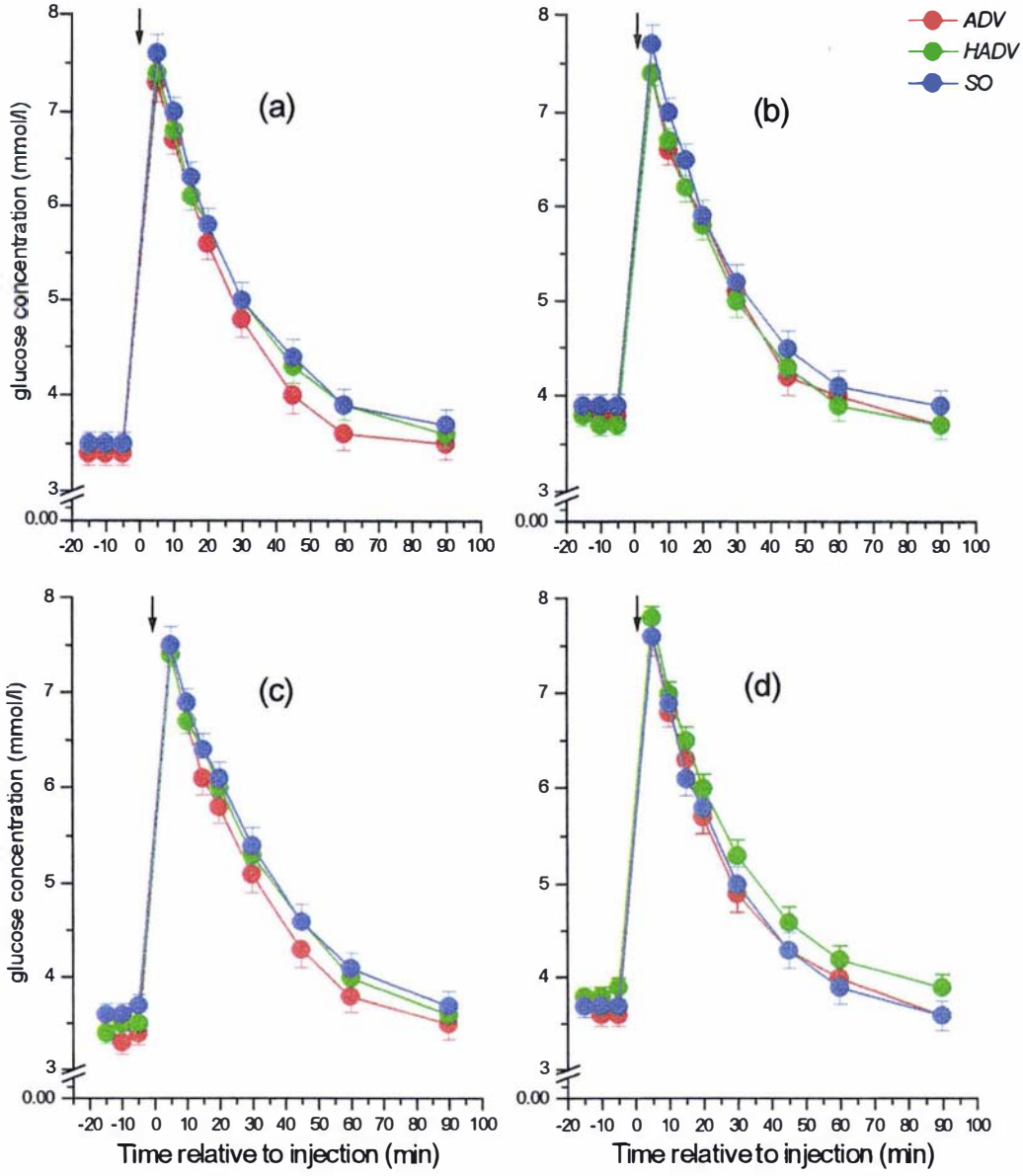

Figure 5.1 Plasma glucose concentrations in abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) wethers before and after intra-jugular glucose injection. Glucose injection 2 h (a) and 22 h (c) after feeding in wethers fed on the low plane of nutrition, and 2 h (b) and 22 h (d) after feeding in wethers fed on the high plane of nutrition. Legends are shown at top right hand corner. Arrow indicates the time of glucose injection. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

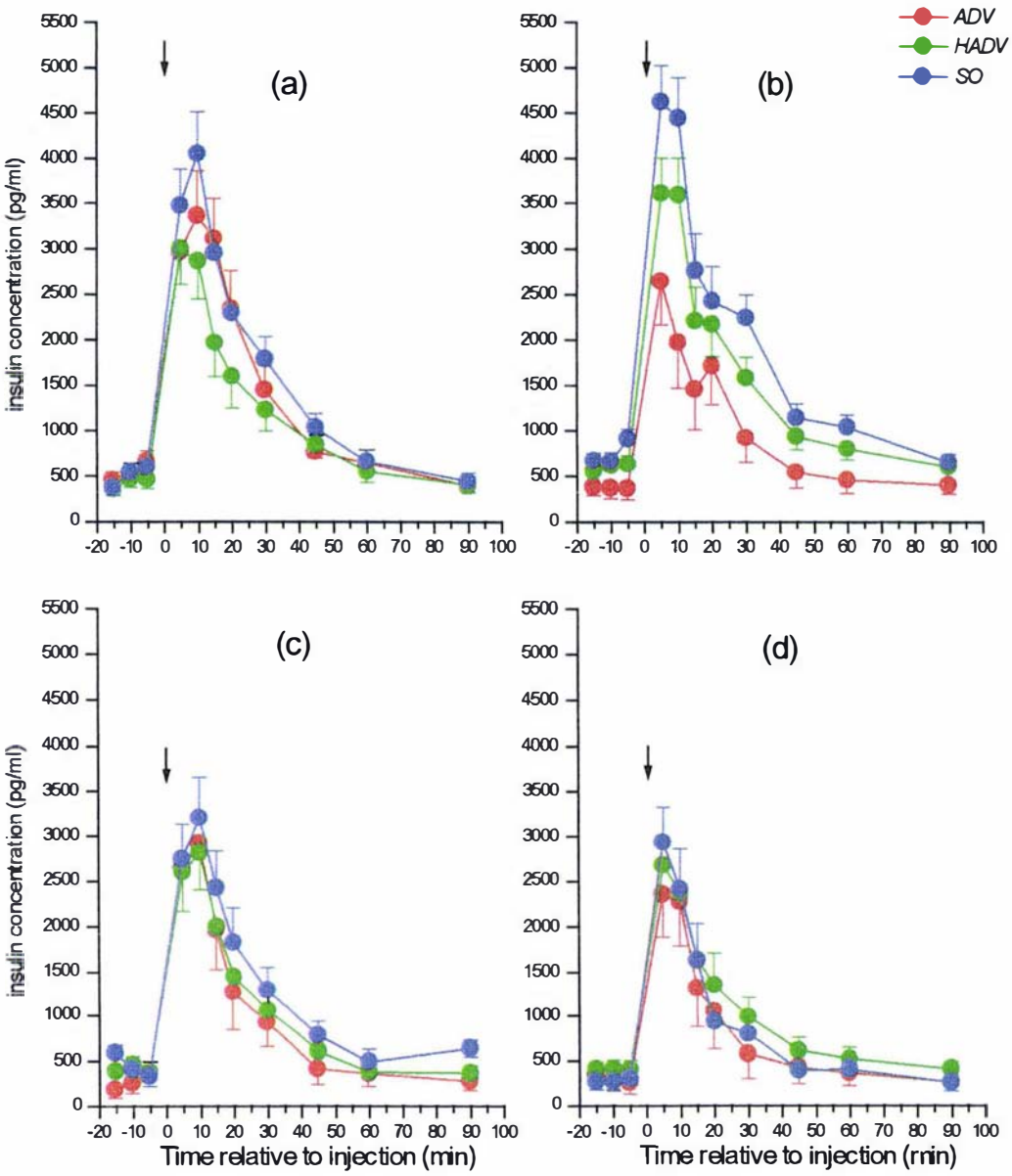

Figure 5.2 Plasma insulin concentrations in abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) wethers before and after intra-jugular glucose injection. Glucose injection 2 h (a) and 22 h (c) after feeding in wethers fed on the low plane of nutrition, and 2 h (b) and 22 h (d) after feeding in wethers fed on the high plane of nutrition. Legends are shown at top right hand corner. Arrow indicates the time of glucose injection. Plasma insulin concentrations were significantly ($P < 0.05$) higher in the SO animals than that in the ADV or HADV animals. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

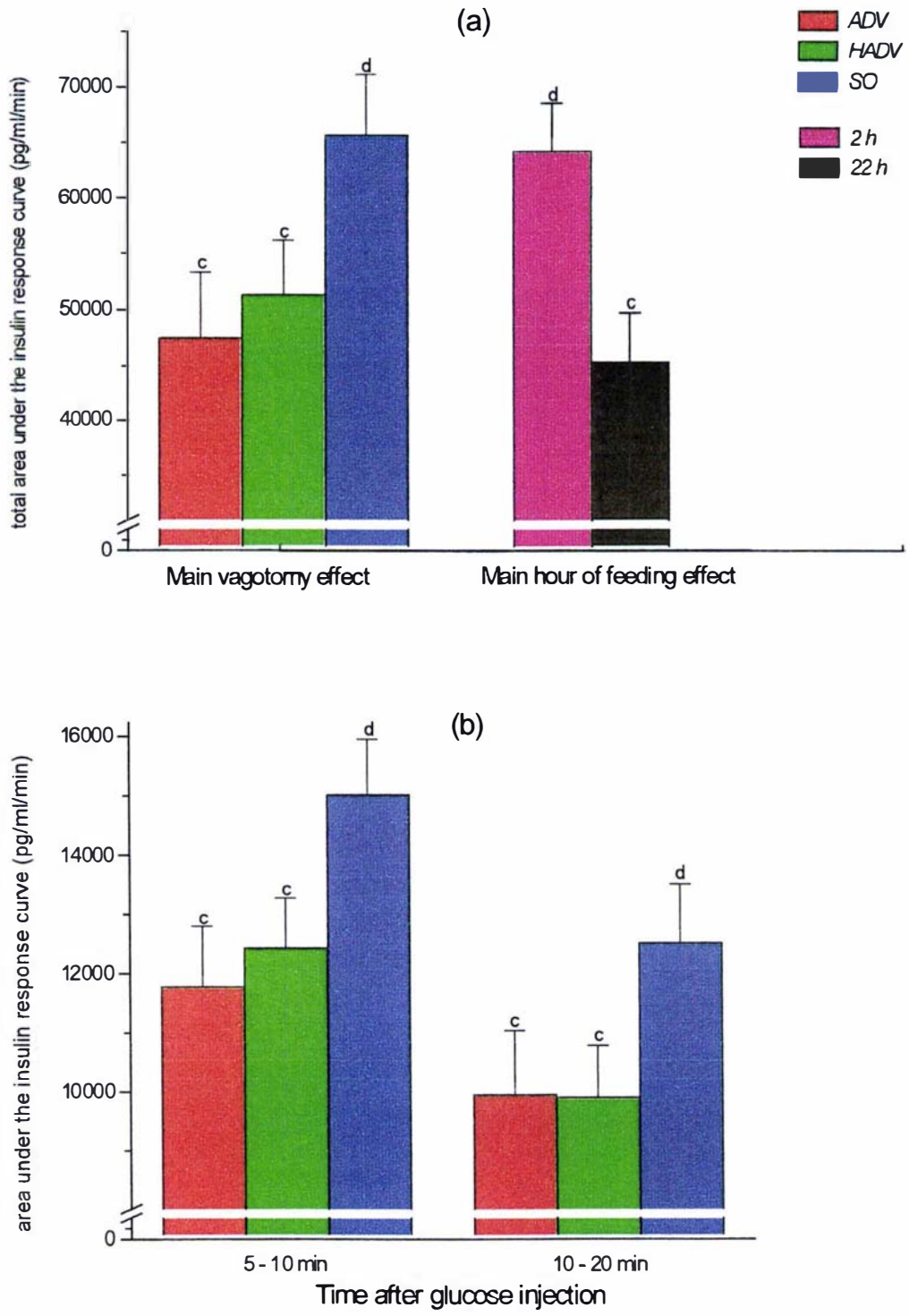

Figure 5.3 (a): The total area under the insulin response curve, corrected for baseline, for 90 minutes in abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) wethers (main vagotomy effect) and in wethers received glucose injection 2 hours (2 h) and 22 hours (22 h) after feeding (main hour of feeding effect). (b): The areas under the insulin response curve, corrected for baseline, for the periods of 5-10 min and 10-20 min post-injection in ADV, HADV and SO groups. Legends are shown at top right hand corner. $^{cd}P < 0.05$, results with different letters are significantly different. Vertical bars: SEM.

5.4.4 Postprandial patterns of plasma glucose and insulin

5.4.4.1 Plasma glucose

Statistical analyses of the data showed that glucose concentrations in wethers that underwent surgery on day 4 (group 4) were significantly ($P < 0.0002$) higher than that of groups 1, 2, and 3 (i.e. days 1, 2 and 3, respectively) at 5, 6, 7, 8, and 9 h post-feeding before crossing-over (see Table 5.1). Glucose concentrations were elevated in all animals in Group 4, irrespective of the treatment or the level of feeding. Such high concentrations after feeding is unusual and may have resulted from the use of glucose contaminated syringes for blood sampling, although the exact cause is not certain. Therefore, the data at 5, 6, 7, 8, and 9 h post-feeding for each animal in group 4 (before cross-over) were excluded from further analyses for plasma glucose only and the remaining data were presented in Figure 5.4.

Plasma glucose concentrations were significantly ($P < 0.05$) higher in wethers fed on the high plane of nutrition than those fed on the low plane of nutrition. Plasma glucose concentrations were not affected by selective vagotomy. The feeding related changes in glucose concentrations were not large, and the pattern of change with time was similar in all treatment groups at both levels of nutrition. In the LP group, the increases in glucose concentrations were small and reached maximum values 6-8 h after eating (Fig. 5.4 (a)). In the HP group, increases in glucose concentrations were also small and reached maximum values 6-8 h after eating (Fig. 5.4 (b)).

5.4.4.2 Plasma insulin

In the LP group, ingestion of food caused small but irregular increases in insulin concentrations in all wethers but the increases were larger in SO wethers, maximum values being reached 8 h after feeding (Fig. 5.4 (c)). Ingestion of larger amounts of food (HP group) was followed by a rapid increase in plasma insulin concentrations in the SO group, maximum values (approximately 100% greater than prefeeding values) being reached 1 h after feeding (Fig. 5.4 (d)). The increases in the ADV and HADV treatment groups were not large and reached maximum values respectively 2-3 and 2-4 h after feeding (Fig. 5.4 (d)).

Plasma insulin concentrations were highly significantly ($P < 0.0002$) suppressed in ADV and HADV wethers, compared with that of SO wethers. The wethers fed on the high plane of nutrition had significantly ($P < 0.05$) elevated insulin concentrations compared with those fed on the low plane of nutrition. Changes in plasma insulin concentrations after feeding varied highly significantly ($P < 0.0002$) with time but were significantly dependent on the vagotomy ($P < 0.02$) and the plane of nutrition($P < 0.05$).

Table 5.1 Differences in mean plasma glucose concentrations (mmol/l) between 4 sampling days, irrespective of the main treatment groups, measured at 5, 6, 7, 8 and 9 h after feeding.

Hours after feeding	Plasma glucose concentration (mmol/l) [Mean ± SEM]				Probability Value
	Day 1	**Day 2**	**Day 3**	**Day 4**	
5	3.7 ± 0.3^{a}	3.6 ± 0.4^{a}	3.7 ± 0.3^{a}	4.9 ± 0.3^{b}	$P < 0.0001$
6	3.7 ± 0.3^{a}	3.7 ± 0.3^{a}	3.7 ± 0.3^{a}	5.4 ± 0.2^{b}	$P < 0.0002$
7	3.8 ± 0.1^{a}	3.6 ± 0.2^{a}	3.7 ± 0.1^{a}	4.8 ± 0.1^{b}	$P < 0.0001$
8	3.8 ± 0.3^{a}	3.8 ± 0.4^{a}	3.8 ± 0.3^{a}	5.8 ± 0.3^{b}	$P < 0.0001$
9	3.8 ± 0.1^{a}	3.6 ± 0.1^{a}	3.8 ± 0.1^{a}	5.5 ± 0.1^{b}	$P < 0.0001$

^{a,b} Means with different superscripts are significantly different. Therefore, these data from Day 4 were excluded from the analyses for postprandial variation of plasma glucose only.

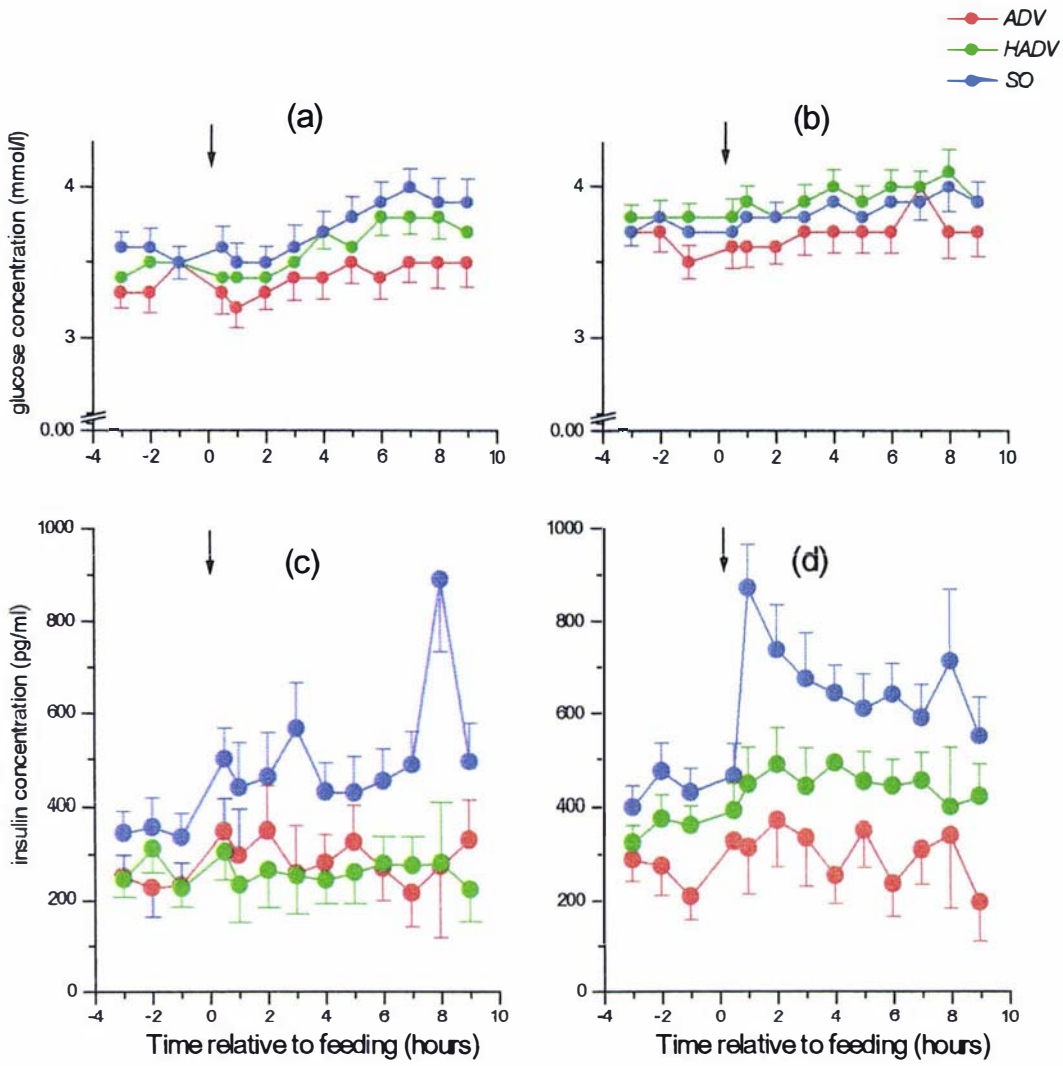

Figure 5.4 Postprandial patterns of plasma glucose and insulin before and after feeding in abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) wethers. Glucose (a) and insulin (c) in wethers fed on the low plane of nutrition and glucose (b) and insulin (d) in wethers fed on the high plane of nutrition. Legends are shown at top right hand corner. Arrow indicates the time of feeding. Plasma insulin concentrations were significantly ($P < 0.0002$) higher in the SO animals than that in the ADV or HADV animals. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

5.4.5 CPIR

5.4.5.1 Plasma glucose

Plasma glucose concentrations were not significantly different between treatment groups (Fig. 5.5 (a)). Further, Duncan's test showed that plasma glucose concentrations were not elevated significantly after feeding in either of the 3 treatment groups during 10 min period.

5.4.5.2 Plasma insulin

Insulin concentrations in one of the ADV animals was found to be extremely high before and after presentation of food. Dixon's test (Fry, 1993) showed that these values were significantly ($P < 0.05$) higher than the rest of values in the treatment group and therefore, they were excluded from the analysis.

Insulin response after start of eating was significantly ($P < 0.05$) suppressed in wethers of ADV and HADV groups while insulin concentrations were significantly ($P < 0.05$) increased in the SO wethers, maximum values (approximately 200% greater than prefeeding values) being reached 4 min after feeding (Fig. 5.5 (b)). The rise of plasma insulin, which occurred almost instantly when food was offered, was maintained at a significantly higher level until 6 min after which it gradually declined to prefeeding values. This resulted in significantly ($P < 0.05$) larger, baseline corrected, areas under the response curve for the periods of 1-2 min, 2-4 min and 4-6 min (Fig. 5.6 (a)) and larger ($P < 0.05$) total area for 10 min (Fig. 5.6 (b)), for the SO group compared with that of both the ADV and HADV groups.

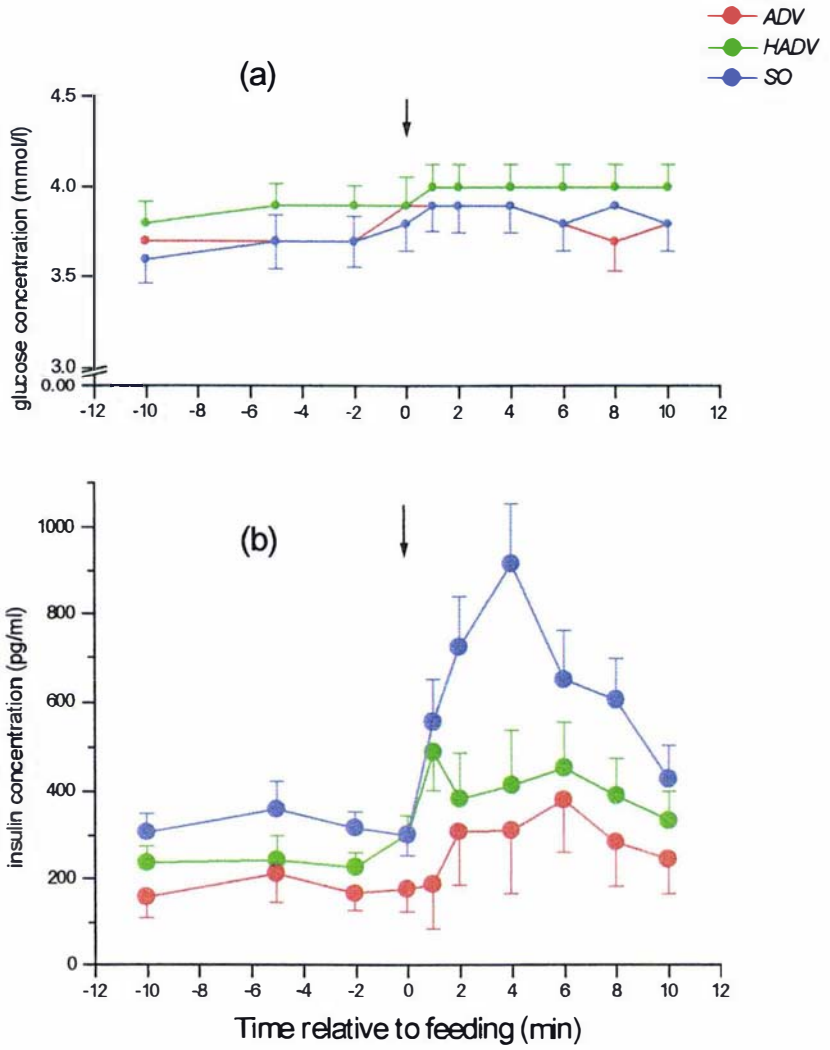

Figure 5.5 Glucose response (a) and cephalic phase insulin release (CPIR) (b) in response to presentation of food in abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) wethers. Legends are shown at top right hand corner. Arrow indicates the time of the presentation of food. Plasma insulin concentrations were significantly ($P < 0.05$) higher in the SO animals than that in either the ADV or HADV animals. Not all error bars are shown to preserve clarity. Vertical bars: SEM.

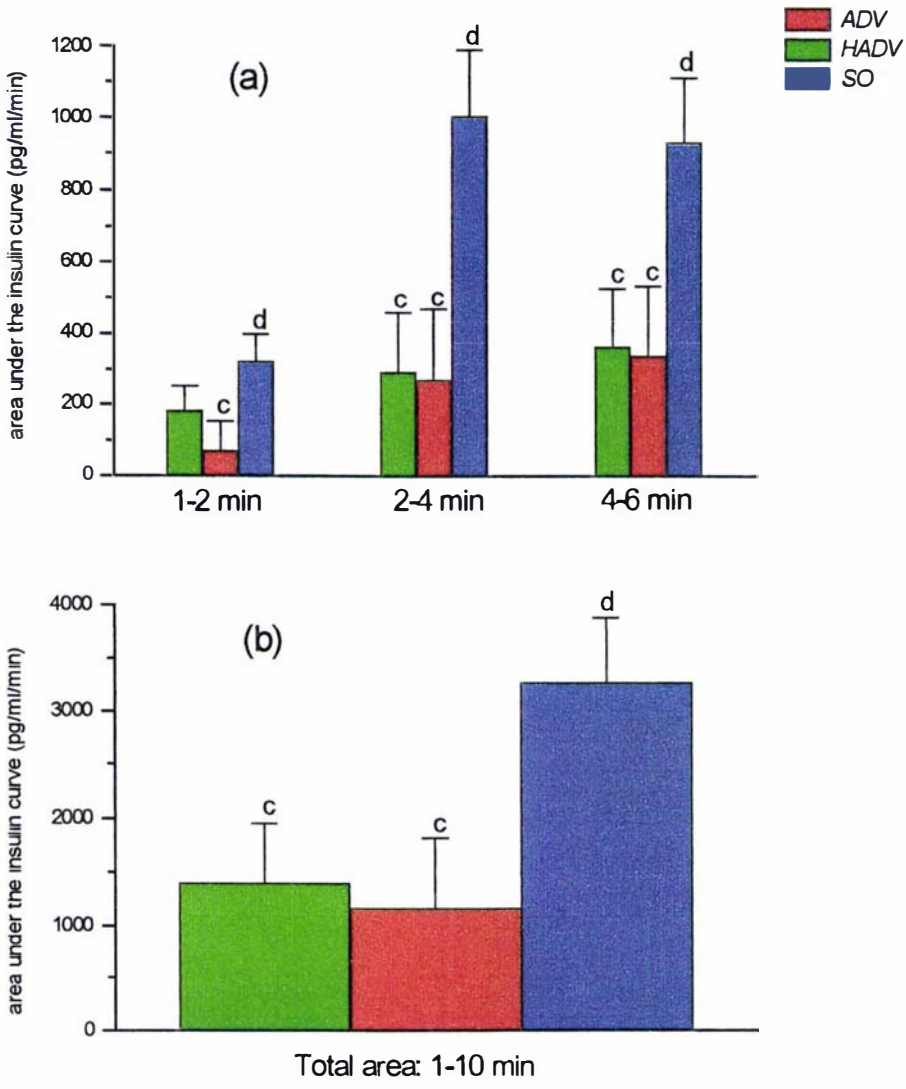

Figure 5.6 Cephalic phase insulin release. The areas under the insulin response curve, corrected for baseline, for the periods of 1-2 min, 2-4 min and 4-6 min (a) and the total area under the insulin response curve, corrected for baseline, for 10 min (b) in response to presentation of food in abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) wethers. Legends are shown at top right hand corner. $^{cd}P < 0.05$, results with different letters are significantly different. Vertical bars: SEM.

5.4.6 Physiological confirmation of vagotomy

Electrical stimulation tests were successful only in the first 3 animals one from each of the 3 treatment groups i.e. ADV, HADV and SO groups. Since the tests for the rest of the animals were unsuccessful due to technical difficulties the data from these animals were not used and thus were not presented. Therefore, Figure 5.7 shows the insulin response in the first 3 animals; data presented as absolute values for each of the 3 animals.

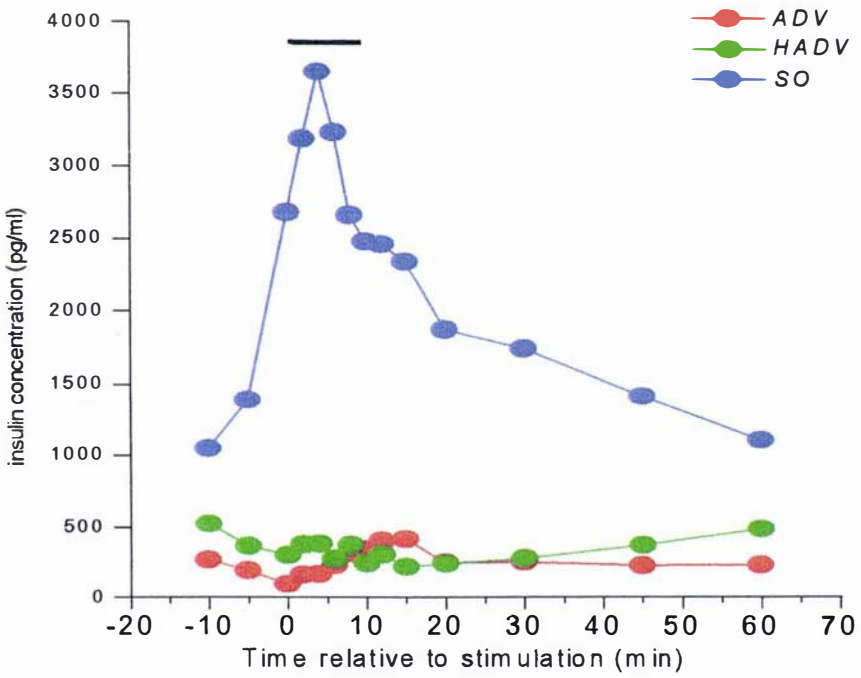

Figure 5.7 Insulin response to bilateral electrical stimulation (using short bursts of 1 sec duration every 10 sec, set at 10 mA and 40 Hz for 10 min) of the peripheral ends of the cut cervical vagus nerves in anaesthetized wethers. Data are presented as absolute values in 3 animals one from each of the 3 treatment groups i.e. abomasal and duodenal vagotomized (ADV), hepatic, abomasal and duodenal vagotomized (HADV) and sham-operated (SO) groups. Legends are shown at top right hand corner. Horizontal thick bar indicates the duration of stimulation.

5.5 DISCUSSION

The present study investigated the role of the vagal innervation of the abomasum, pylorus, duodenum and liver on insulin release in response to intravenous glucose injection at two different times in relation to feeding at two levels of nutrition and also in response to electrical stimulation of the cervical vagus nerves in wethers. This study also investigated postprandial patterns of plasma glucose and insulin and the cephalic phase insulin release in response to feeding.

Plasma insulin release in response to intra-jugular glucose injection was suppressed in wethers in which the abomasal (including pylorus) and duodenal (ADV) or the hepatic, abomasal (including pylorus) and duodenal (HADV) branches of the vagus nerves were cut (Figure 5.2). Thus the baseline corrected total areas under the insulin response curve for each of the two groups of vagotomized animals were significantly lower than that of sham-operated animals (Figure 5.3 (a)). This is in line with earlier findings (Chapter 2) that abomasal and duodenal vagotomy suppressed plasma insulin secretion in response to glucose injection in lactating ewes.

The insulin secretion was greatly influenced by the vagal innervation of the GI tract in lactating ewes (Chapter 2). Thereafter, the response to vagotomy in previous experiments, however, has been variable. The plane and the pattern of nutrition were considered to be important for pancreatic β-cell sensitivity though vagal neurons in the GI tract. It was further suggested (Chapter 4) that insulin release in response to glucose injection may be modulated by the vagal innervation of the GI tract but the extent of which can be varied and dependent on the degree of β-cell sensitivity at the time of glucose injection. The food in the GI tract initiates vagal signals and sensitises β-cells. In the present experiment, the plane of nutrition, the time of glucose challenge relative to time of feeding and the time after surgery were expected to alter nutrient availability and hence the β-cell sensitivity and the response to glucose injection.

Insulin secretion was significantly higher in response to glucose injection administered 2 h after feeding compared with that 22 h after feeding (Figures 5.2 and 5.3 (a)), indicating that the magnitude of insulin response is influenced to a greater extent by

the presence of food in the GI tract and perhaps the amount of food ingested. This was also reflected in the significantly increased rates of glucose removal in wethers receiving a glucose injection 2 h after feeding. There was a general, although not significant, trend for higher insulin concentrations in animals fed on the high plane of nutrition compared with those fed on the low plane of nutrition when glucose injection was given 2 h after feeding (Figure 5.2 (a),(b)). This was also reflected in the significantly reduced rates of glucose removal in wethers fed on the low plane of nutrition. The greater response to glucose injection 2 h after feeding, in particular in those wethers fed on the high plane of nutrition, is consistent with the pattern of postprandial insulin release observed.

The greater insulin response 9 days after the first set of glucose challenges was consistent with earlier findings that the greater response was due to a higher energy balance at this time in wethers. Thus the neural signal the pancreas gets from the GI tract is overwhelmed by the magnitude of the stimulus that the pancreas gets from the systemic circulation in the form of nutrients. The availability and also the amount of nutrients in the GI tract were major determinants in the extent of the β-cell sensitivity through vagal inputs from the GI tract and hence the response to acute glucose challenges. Postprandial insulin concentrations were significantly increased after eating in wethers fed on the high plane of nutrition than those fed on the low plane of nutrition (Figure 5.4 (c),(d)). This reflects the observed difference in the magnitude of insulin response to glucose injection between two feeding levels (Figure 5.2 (a),(b)). The higher postprandial insulin concentrations were apparent in the sham-operated animals whereas changes in concentrations in both groups of vagotomized animals were small and nonsignificant. This is consistent with findings that insulin concentrations were higher in the sham-operated animals following glucose injection than those of the vagotomized animals. Furthermore the elevated insulin concentrations within the first 3 h following feeding, particularly in wethers fed on the high plane of nutrition, also suggest that insulin release in response to glucose injection could be amplified if administered at this time after feeding. This is supported by findings that an increased release of insulin in response to glucose injection occurred when it was administered 2 h after feeding.

The increased plasma insulin concentrations in sham-operated animals fed on the high plane of nutrition started to decline 4 h after eating. The postprandial variation of

plasma insulin concentrations during a 9 h post-feeding period reported in this study is similar to that reported in sheep (Trenkle, 1970) and heifer calves and steers (McAtee and Trenkle (1971). Trenkle (1970) observed that when sheep were fed on hay or grain, peak plasma insulin concentrations occurred 4 h after feeding and then gradually decreased. In the present study, plasma insulin concentrations were higher and maintained for longer after eating in sham-operated wethers fed on the high plane of nutrition than those fed on the low plane of nutrition. This agrees with findings of Bassett (1971) who reported that plasma insulin concentrations are positively correlated to digestible nutrient intake in sheep. This supported the findings of Trenkle (1970) who showed that plasma insulin concentrations were increased with increasing amounts of digestible energy intake in sheep. In the study of Bassett (1974b) a rapid increase in plasma insulin occurred after eating, but the rise was not accompanied by a significant increase in plasma glucose concentrations which always occurred later and more slowly than those in insulin. They concluded that changes in plasma glucose concentrations play little part in determining the initial increase in insulin concentrations, although the eventual increase in glucose concentrations may play some part in the maintenance of the high insulin levels. They further suggested that increases in insulin secretion after feeding may be regulated by other mechanisms and the most likely candidates are gastrointestinal hormones secreted during feeding in sheep. However, results in the present study clearly indicate that the feeding related rise of plasma insulin concentrations is a consequence of the direct effect of the vagal innervation of the GI tract in sheep. Therefore, the results suggest that vagus nerves play a major role in initiating and possibly also in maintaining the insulin secretory response to feeding in sheep.

Ingestion of smaller or larger amounts of food resulted in increases in plasma glucose concentrations in all animals, but the increases were small and not significantly different between treatment groups. The findings that small changes in postprandial plasma glucose concentrations in both levels of feeding, is consistent with earlier findings in sheep (Bassett, 1974b; Trenkle, 1970). However, in the present study, plasma glucose concentrations were significantly higher in wethers fed on the high plane of nutrition than those fed on the low plane of nutrition (Figure 5.4 (a),(b)). There was no direct relationship found between plasma glucose and insulin concentrations, which agrees with

the findings of other workers (Manns and Boda, 1967; Horino *et al.*, 1968; Trenkle, 1970; McAtee and Trenkle, 1971; Ross and Kitts, 1973). It is known that plasma glucose concentration is maintained within a relatively narrow range in ruminants. The findings of Bassett (1974b) are similar to findings of this study in which plasma glucose concentrations reached maximum values 6 to 8 h after eating in all intake groups. The gradual increase in glucose concentrations observed 6 to 8 h after eating in the present study was probably due to an increased rate of gluconeogenesis (Bergman and Wolff, 1971) and increased plasma glucagon concentrations (Bassett, 1972).

The preabsorptive release of insulin in response to the presentation and/or ingestion of food (Figure 5.5 (b)), the cephalic phase secretion, is well documented in non-ruminants (Strubbe and Steffens, 1975; Strubbe, 1992; LeBlanc *et al.*, 1991; Berthoud *et al.*, 1980, 1981; Berthoud and Powley, 1990; Berthoud and Jeanrenaud, 1982; Steffens, 1976; Rogers and Blundell, 1989; Yamazaki and Sakaguchi, 1986; Bruce *et al.*, 1987; Teff *et al.*, 1993, 1995) and ruminants (Faverdin, 1986b; Porter and Bassett, 1979; Bassett, 1974a; Lofgren and Warner, 1972; Vasilatos and Wangsness, 1980; Bhattacharya and Alulu, 1975; Chase *et al.*, 1977). The observation (Figure 5.5 (b)) that vagotomy suppresses the secretion of insulin that occurs immediately after the start of eating, has not been reported previously in a ruminant. The baseline corrected area at 2-4 and 4-6 minutes (Figure 5.6 (a)) and the total area (Figure 5.6 (b)) under the insulin response curves after feeding for the SO and both the vagotomized groups were significantly ($P < 0.05$) different. The rapidity of the insulin response, within 2-4 minutes following the start of food ingestion in the sham-operated animals, together with no increase in plasma glucose concentrations, suggest that the stimulus for insulin release was neural in origin. A purely humoral mediation of this rapid insulin response has also been ruled out by β-cell transplantation experiments (Berthoud *et al.*, 1980; Strubbe and Wachem, 1981).

Lofgren and Warner (1972) have observed a rapid increase in insulin concentrations in sheep within 15 minutes after feeding without any change in glucose concentration. These results agree with the findings of Bassett (1974a) that the ingestion of food caused a very rapid increase in insulin response within 5 minutes in adult sheep and lambs. Later Chase *et al.* (1977) reported similar findings in a study with steers in which insulin release was rapid and increased significantly within the first 6 minutes of the

ingestion of food. Chase *et al.* (1977) and Bassett (1974a) suggested the involvement of a neural reflex pathway in the regulation of the rapid increase in plasma insulin because the changes in insulin were rarely accompanied by changes in metabolite concentrations. Thus, the results of the present study clearly show that this rapid increase in plasma insulin immediately after feeding was not accompanied by changes in blood glucose concentrations. Furthermore, these data that indicate an effect of vagotomy on CPIR in wethers are strongly supported by results from the literature, both in non-ruminants (Strubbe, 1992; Berthoud and Powley, 1990; Berthoud *et al.*, 1981,1982) and ruminants (Porter and Bassett, 1979) and were discussed in Section 1.4.4.

Furthermore, the involvement of a reflex centre in the hypothalamus in the mediation of food-related CPIR has been reported (Niijima, 1991; Niijima *et al.*, 1990). Niijima *et al.* (1990) reported that an application of monosodium glutamate to the tongue in the anaesthetized rats caused a significant increase in efferent discharge rate of the pancreatic branch of the vagus nerve, which commenced 5 minutes after the stimulation and lasted for about 30 minutes. This increased efferent activity was found to coincide with cephalic phase insulin secretion in these rats. The results were further supported by the findings of Niijima (1991) that an application of 10% sucrose to the tongue caused a significant increase in efferent activity of the pancreatic branch of the vagus nerve in an anaesthetized rat. He also observed a similar response in the decerebrated rats, indicating the existence of the reflex centre in the hypothalamus. Niijima (1991) suggested that oral stimulations, as used in these experiments, through afferent neurons, activate efferent parasympathetic neurons in the DMN of the vagus in the brain stem , which innervate the pancreatic β-cells and in turn cause the cephalic insulin release. Therefore, in the present experiment, cephalic phase insulin release observed in the sham-operated wethers was most likely a consequence of the direct vagal signal that was carried from the hypothalamus to pancreatic β-cells via vagal efferent fibres in the abomasal, pyloric and duodenal branches of the vagus nerves (see Figure 1.3). Further, although the anatomical location of the branch of the vagus that innervates the pancreas in ruminants is not clear, a possible pathway through the gastric branches has been reported (Kirchgessner and Gershon, 1990, 1989; Poulsen *et al.*, 1983; Matsuo and Seki, 1978; Tiscornia, 1977; Stavney *et al.*, 1963) and discussed in Section 1.4.3.1.

The terminal acute experiment in which both cervical vagus nerves were electrically stimulated was successful only in the first three animals, one from each of the three treatment groups, due to technical difficulties. It was evident from results of these three wethers that insulin secretion was stimulated in the sham-operated animal but not in the abomasal and duodenal vagotomized and hepatic, abomasal and duodenal vagotomized animals (Figure 5.7), presumably because the vagal innervation was cut in the latter. Thus, the results in Chapter 4 together with those from the present experiment indicate that the vagal innervation to the GI tract had been successfully cut in these animals.

The various responses measured, including the suppression of insulin release, in both the ADV and HADV groups were all similar in magnitude (Figures 5.2 to 5.7) which indicates that hepatic, abomasal and duodenal vagotomy did not have any additive effect over that achieved by the abomasal and duodenal vagotomy alone. Therefore, the findings indicate that the hepatic branch is not implicated in these types of insulin release and thus the vagal fibres contained in the abomasal, pyloric and duodenal branches of the vagus nerves are solely responsible for eliciting the insulin responses during hyperglycaemia, feeding and cephalic phase in sheep.

In conclusion, these results suggest that the vagal innervation of the GI tract modulates pancreatic insulin secretion in response to intra-jugular injection of glucose in wethers. The results support the concept that the suppression of insulin release in response to glucose injection is a general response to cutting the vagal innervation to the GI tract and the effect is not confined to lactating ewes but also occurs in wethers. It provides further support for the hypothesis that the magnitude of the plasma insulin response during hyperglycaemia is dependent on the degree to which the β-cells have been sensitised by vagal neurons activated by the presence of food in the GI tract. Therefore, it is suggested that when food is available in the GI tract, and also the amount food available is large, the stimulus the pancreatic β-cells get from the GI tract in the form of vagal inputs overrides other possible stimuli such as stimulus from the systemic circulation in the form of nutrients. It is also suggested that insulin secretion after feeding is regulated in part by the vagal nerves and that blood glucose levels may not play an exclusive role in the regulation of insulin secretion. The results further suggest that feeding-associated cephalic phase

insulin release is also mediated by neurons contained in the abomasal, pyloric and duodenal branches of the vagus nerves in wethers.

CHAPTER 6

THE VAGAL CONTROL OF SUCKLING-ASSOCIATED OXYTOCIN AND INSULIN RELEASE IN LACTATING EWES

6.1 ABSTRACT

The role of the vagal innervation of the abomasum, pylorus and upper small intestine in the release of OT during suckling with or without simultaneous feeding, was studied in lactating ewes. Insulin release during suckling and in response to presentation of food was also studied. Abomasal and duodenal vagotomy (ADV) was carried out in 5 ewes and 5 ewes underwent sham-operations (SO). After surgery they were fed fresh lucerne at 1.8 x daily energy requirements for maintenance as estimated from body weights. Food was offered once a day at 1100 h. Suckling-associated OT release without simultaneous feeding was measured on PO day 8. The timing of sucking by the lambs was controlled by returning the lambs to their dams after a 3 h isolation period. Three discrete syringe blood (5 ml) samples 10, 5 and 2 min before suckling started and continuous blood (5 ml) samples using a peristaltic pump 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, 9-10 min and the last discrete syringe sample 15 min after suckling started were drawn. On PO day 9, the same procedure was repeated but the ewes were offered fresh lucerne 15 min before the lambs were returned to the pens and they were allowed to eat while nursing their lambs. These blood samples were analyzed for plasma OT and the samples taken from the experiment on PO day 8 were also analyzed for plasma insulin. On PO day 12, ewes were offered fresh lucerne and blood samples (5 ml) were collected at -10, -5, -2, 1, 2, 4, 6, 8, 10 min relative to time of the presentation of food to investigate the CPIR. Samples were analyzed for plasma glucose and insulin. Suckling-associated OT concentrations were generally higher in the SO than in the ADV ewes and also during simultaneous suckling and feeding than that during suckling without simultaneous feeding as reflected in the baseline corrected total area under the OT curve, but the differences were not statistically significant. However, the concentrations were increased significantly ($P < 0.05$) at 3-4 min of suckling from baseline concentrations in the sham-operated ewes fed and suckled simultaneously. Presentation of food caused a significant ($P < 0.05$) rise in plasma insulin concentrations from its baseline values in the SO ewes but not in the ADV ewes. Baseline corrected areas under the insulin response curve immediately following feed presentation such as 1-2 and 2-4 min, were significantly ($P < 0.05$) lower in the ADV than in the SO ewes. Insulin release during suckling tended to be lower ($P < 0.09$) in the ADV than in the SO ewes. These results indicate that the vagal innervation of the GI tract

plays a major role in the cephalic phase insulin release in response to presentation of food in lactating ewes and confirm the previous results in wethers. The results further suggest that vagus nerves are important in the release of insulin during suckling in lactating ewes. The findings of the present study in lactating ewes do not, however, completely confirm the findings that vagotomy inhibits suckling-stimulated OT release in rats. However, based on these findings together with the previous results (Chapter 4), it is very likely that OT release in response to suckling and also simultaneous suckling and feeding is modulated by the vagus nerves in ewes.

6.2 INTRODUCTION

In a previous study (Chapter 4) suckling-associated OT release was investigated in lactating ewes. This study indicated that OT concentrations before suckling were significantly reduced in the vagotomized ewes, but OT release during suckling was unaffected. However, blood samples were drawn at infrequent intervals so that peak OT concentrations may have gone undetected in the sham-operated animals. Therefore, the present experiment was designed to investigate the OT response during suckling in sham-operated and vagotomized ewes using a procedure that facilitated the collection of more frequent blood samples during suckling.

The results from the previous experiment (Chapter 5) indicated that the vagus nerve plays a major role in the CPIR in response to presentation of food in wethers. Therefore, since insulin release was suppressed by vagotomy in response to presentation of food in wethers (Chapter 5) and also to intrajugular glucose injection in lactating ewes (Chapter 2) and wethers (Chapter 5), it was of interest to investigate the effect of vagotomy on CPIR in response to presentation of food in the present experiment in lactating ewes.

Insulin has been shown to be released in response to suckling in several species including the human being (Franceschini *et al.*, 1990; Eriksson *et al.*, 1987; Uvnas-Moberg *et al.*, 1984; Widstrom *et al.*, 1984; Uvnas-Moberg and Eriksson, 1983). Although the mechanism regulating suckling-associated release of insulin is not known, these authors proposed that suckling-induced insulin release is secondary to a reflex

activation of vagal neurons since the release of insulin is under vagal control. Therefore, since the release of pancreatic insulin under various physiological conditions is greatly influenced by the vagal innervation of the GI tract in sheep (Chapters 2, 4 and 5), it was of interest to investigate the role of the vagus nerve in the release of insulin during suckling in lactating ewes.

The experiments described in this chapter were designed to demonstrate the effects of cutting the vagal innervation to the abomasum, pylorus and duodenum on suckling-associated OT release with or without simultaneous feeding and also of insulin release in response to suckling and the presentation of food in lactating ewes.

6.3 MATERIALS AND METHODS

6.3.1 Animals, housing and acclimatisation

Twenty late pregnant Romney ewes were obtained from AgResearch farms, Palmerston North. They were grazed on pasture until lambing. After lambing they were housed indoors, each with a single lamb, in metabolism cages. One of each twin born lamb was fostered. Ewes and lambs were returned to pasture after the experiment.

Ewes were accustomed to housing, handling, feeding and other routine maintenance procedures and given water *ad libitum* at all times. They were fed fresh lucerne (12 MJ ME/kg DM) *ad libitum* over the one week acclimatisation period. Food was offered twice a day at 0900 and 1900 h. Ten of those that adapted and were eating well were selected for the subsequent experiment. Approximately one week after lambing all animals underwent surgery and were randomly allocated to either selectively vagotomized or sham-operated groups (for details see Section 6.3.3). All procedures involving ewes were approved by the AgResearch Animal Ethics Committee.

6.3.2 Surgical procedures

Surgery was carried out as described in section 2.3.2.1 (see also No. 1, 2 & 3 of Figure 1.1). In the present experiment, the abomasal and pyloric branches of the dorsal vagus nerve were also sectioned (see No. 5 & 6 of Figure 1.2). Jugular catheters were inserted as described in Section 2.3.2.2.

6.3.3 Experimental design

Surgery was carried out over two days. On the first day, 5 randomly selected ewes underwent abomasal and duodenal vagotomy (ADV; n=2) or sham-operation (SO; n=3), while on day 2 the remainder underwent surgery (ADV; n=3, SO; n=2). After surgery, ewes were fed at 1.8 x daily energy requirements for maintenance as estimated from body weights (Rattray, 1986). Each treatment group was allocated five animals. The mean (±SEM) body weights at the time of surgery were 55.5 ± 2.4 and 57.4 ± 2.5 kg in the ADV and SO groups, respectively.

6.3.3.1 Suckling-associated OT release

The experiment was carried out one week after surgery.

a) Suckling without simultaneous feeding

Lambs were separated from their mothers for 3 h. Three blood samples (5 ml) were taken 10, 5 and 2 min (i.e. -10, –5 and -2 min relative to time of start of suckling) before the return of the lambs to measure baseline concentrations of OT. Lambs were then brought back to their mothers and allowed to suckle. After collection of the third baseline sample, the jugular catheters were connected to peristaltic pumps (Mode 302F, Watson-Marlow Limited, Falmouth, Cornwall, TR11 4RU, England) and blood was collected continuously for 10 min from the time the lambs were returned, with individual samples (approximately 5 ml) taken over 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9 and 9-10 min. This was followed by a sample taken at 15 min relative to start of suckling.

b) Suckling with simultaneous feeding

The same procedure, as used for the previous experiment, was repeated the next day but, in addition, the ewes were offered fresh lucerne 15 min before the lambs were brought into the pens and they were allowed to eat while nursing their lambs.

Samples were analyzed for plasma OT. In addition, samples collected from the experiment 'a' above were also analyzed for plasma insulin.

6.3.3.2 CPIR

This experiment was carried out on PO day 10. Fresh lucerne was kept in plastic buckets out of the sight of ewes until the time of feeding. Blood samples (5 ml) were taken 10, 5 and 2 min before food was offered. Food was then offered suddenly but quietly and blood samples taken at 1, 2, 4, 6, 8, and 10 min relative to time of presentation of food.

6.3.4 Blood processing and sample analyses

All blood samples were collected from a jugular catheter into 0.05 ml of 15% Na_2 EDTA (1.5 mg per ml blood) to prevent clotting. The samples were stored in ice and centrifuged at 4000 rpm for 15 min at 4° C immediately after completing the experiment. The resulting plasma was stored at -20^0 C, until analysed for plasma glucose, insulin and OT.

Plasma concentrations of glucose and insulin were determined as described previously (Section 2.3.4). Oxytocin concentration was determined after extraction using Sep-Pak-Plus C_{18} cartridge as described in the appendix 1. Oxytocin results were not corrected for incomplete recovery since only the comparisons between treatment groups were made.

6.3.5 Statistics

Statistical significance between the means of treatment groups was determined using ANOVA designed to account for repeated measures. Duncan's multiple comparison range test was used to compare the means within individual treatment groups when the time and the interaction between time and treatment effects within subjects were significant. Area under the response curve was determined for data from CPIR and suckling-associated OT release. Data are presented as least square means ± standard errors (±SEM). A probability value less than 0.05 was considered significant. Statistical analyses were carried out using the computer packages 'SAS' (The SAS System for Windows, Version 6.11, SAS Institute Inc., Cary, NC, U.S.A.).

6.4 RESULTS

6.4.1 General effects of vagotomy

It took 24-48 h following surgery for all ewes to return to pre-surgical levels of food intake. Body temperature was normal before and after surgery.

6.4.2 Suckling-associated insulin release

Plasma samples from those animals that were not fed during the suckling experiment were analyzed for insulin concentrations. The suckling-stimulated rise in insulin concentration was rapid and transient in SO ewes. It occurred within 1 min and concentrations returned to baseline within 5 minutes. Suckling-stimulated increases in insulin concentrations in the SO ewes tended to be higher ($P < 0.09$) than those of ADV ewes (Fig. 6.1). However, the concentrations in the ADV ewes were not apparently affected by suckling.

6.4.3 Suckling-associated OT release

Lambs started to suckle their mothers as soon as they were returned to their pens. Ewes started to eat immediately after food was offered. Suckling-associated OT concentrations were not significantly affected by either vagotomy or feeding (Fig. 6.2 (a)). Although the total area under the OT response curve, corrected for baseline, was higher in response to suckling and also suckling with simultaneous feeding in the SO ewes than in the ADV ewes (Fig. 6.2 (b)), the differences were not statistically significant, reflecting the large variation in OT concentrations among the ewes. However, OT concentrations at 3-4 min following suckling were significantly ($P < 0.05$) higher than that of baseline concentrations at -10, -5 and -2 min before suckling in SO animals fed and suckled simultaneously (Fig. 6.2 (a)).

6.4.4 CPIR

6.4.4.1 Plasma glucose

Plasma glucose concentrations were not significantly different between treatment groups (Fig. 6.3 (a)). Further, Duncan's test showed that plasma glucose concentrations

were not elevated significantly after feeding from their before feeding values in either of the 2 treatment groups during the 10 min post-feeding period.

6.4.4.2 Plasma insulin

Presentation of food caused a rise in plasma insulin concentrations to much higher levels in the SO ewes compared with that of ADV ewes, although the overall treatment effect was not statistically significant. However, ANOVA for within subject effects showed significant ($P < 0.05$) effects of time and the interaction between time and vagotomy. Therefore, Duncan's multiple range test was used to compare the means before and after feeding within each treatment group. The test showed that plasma insulin concentrations in the SO ewes rose significantly ($P < 0.05$) to higher levels 2, 4 and 6 min after presentation of food compared with the concentrations 2, 5 and 10 min before presentation of food (Fig. 6.3 (b)). Insulin concentrations in the ADV ewes, however, did not rise significantly.

The rise of plasma insulin, which occurred almost instantly when food was offered, was maintained at a significantly higher level until 6 min after which it gradually declined to prefeeding values. This resulted in significantly ($P < 0.05$) larger, baseline corrected, areas under the response curve for the periods of 1-2 min and 2-4 min and the total area being tended to be larger ($P < 0.10$) for 10 min, for the SO group compared with that of the ADV group (Fig. 6.4).

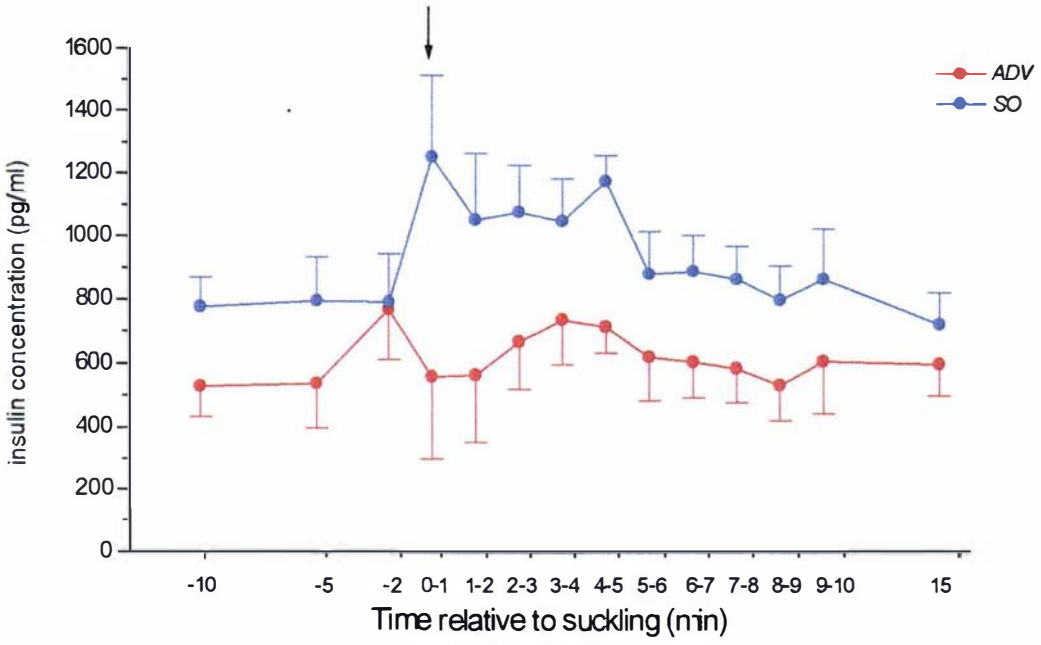

Figure 6.1 Plasma insulin concentrations before and during suckling in abomasal and duodenal vagotomized (ADV) and sham-operated (SO) ewes not fed during suckling. Arrow indicates the time the lambs were returned to their dams after a 3 h separation period. Legends are shown at top right hand corner. X axis labels are shown separately for single time point samples (i.e. -15, -10, -2 and 15 minutes relative to start of suckling) or continuous samples (i.e. 0-1 to 9-10 minutes after start of suckling). Vertical bars: SEM.

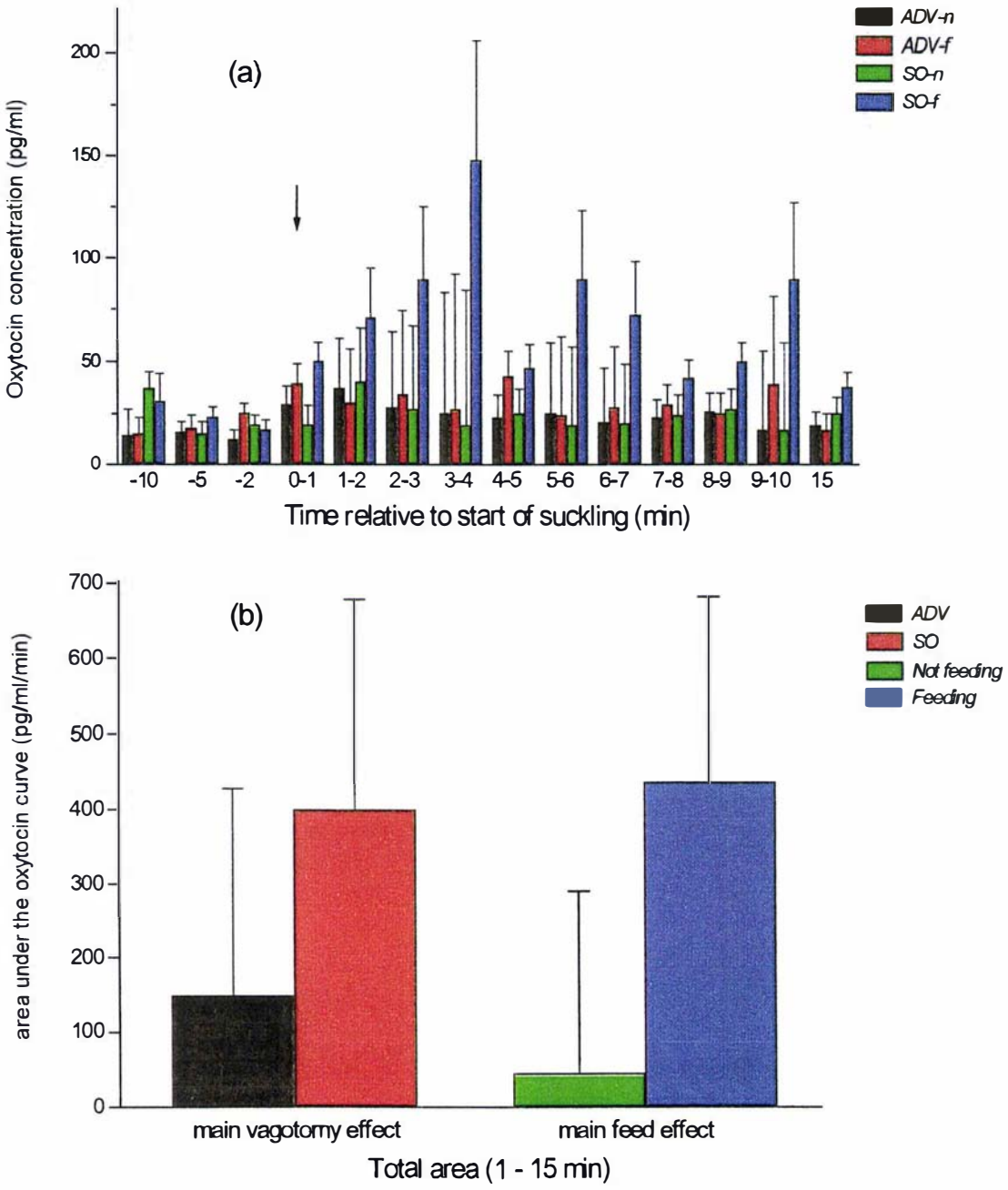

Figure 6.2 (a): Plasma oxytocin concentrations before, and during, suckling in abomasal and duodenal vagotomized (ADV-n) and sham-operated (SO-n) ewes without simultaneous feeding, and in abomasal and duodenal vagotomized (ADV-f) and sham-operated (SO-f) ewes with simultaneous feeding. (b): Total area under the oxytocin response curve during suckling for 15 minutes, corrected for baseline, in the ADV and SO ewes (main vagotomy effect), and during suckling with (Feeding) or without (Not feeding) simultaneous feeding (main feed effect) in ewes. Legends are shown at top right hand corners of panel (a) & (b). X axis labels of panel (a) are shown separately for single time point samples (i.e. -15, -10, -2 and 15 min relative to start of suckling) or continuous samples (i.e. 0-1 to 9-10 min after start of suckling). Arrow in panel (a) indicates the time the lambs were returned to their dams after a 3 h separation period Vertical bars: SEM.

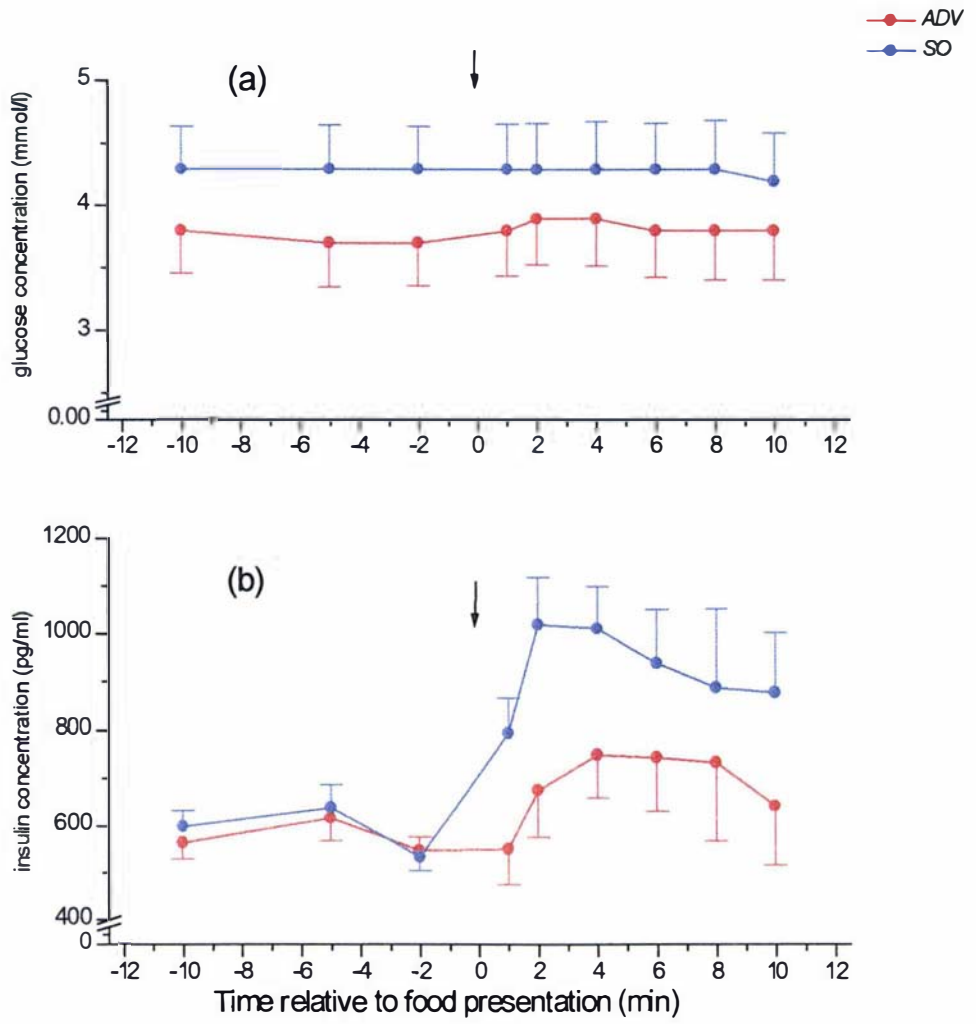

Figure 6.3 Glucose response (a) and cephalic phase insulin release (CPIR) (b) in response to presentation of food in abomasal and duodenal vagotomized (ADV) and sham-operated (SO) ewes. Plasma insulin concentrations were significantly ($P < 0.05$) elevated from their baseline values in the SO ewes but not in the ADV ewes (b). Arrow indicates the time of the presentation of food. Legends are shown at top right hand corner. Vertical bars: SEM.

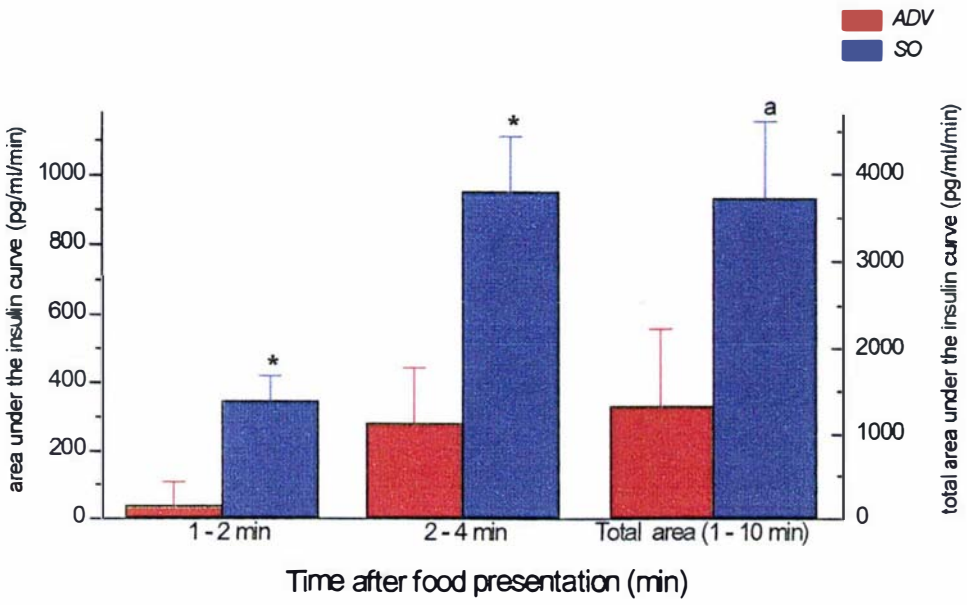

Figure 6.4 The areas under the cephalic phase insulin release (CPIR) curve, corrected for baseline, for the periods of 1-2 and 2-4 min (scaled on the left Y axis) and the total area for 1-10 min (scaled on the right Y axis) in response to presentation of food in abomasal and duodenal vagotomized (ADV) and sham-operated (SO) ewes. $^{a}P < 0.10$, $^{*}P < 0.05$, significance of differences between treatment groups. Legends are shown at top right hand corner. Vertical bars: SEM.

6.5 DISCUSSION

The present study investigated the role of the vagal innervation of the abomasum, pylorus and upper small intestine (duodenum) in the release of OT during suckling with or without simultaneous feeding in lactating ewes. The effect of vagal denervation on insulin release in response to suckling and cephalic phase of feeding was also investigated.

Oxytocin release in response to either suckling or simultaneous suckling and feeding were not significantly different between the vagotomized and sham-operated ewes (Figure 6.2 (a)). However, although not statistically significant, OT concentrations were apparently higher in the sham-operated ewes than in the vagotomized ewes, and were also higher during simultaneous suckling and feeding than during suckling without feeding (Figure 6.2 (b)). Furthermore simultaneous suckling and feeding significantly increased OT concentrations from the baseline concentrations only in the sham-operated ewes. Eriksson *et al.* (1994) demonstrated that suckling-associated OT release was significantly

decreased in vagotomized rats compared to that of control rats, suggesting that suckling-associated OT release is mediated through the vagus nerve. This agrees with findings of others from the literature (Samuelsson *et al.*, 1996b; Svennersten *et al.*, 1995; Svennersten *et al.*, 1990; Stock and Uvnas-Moberg, 1988; Renaud *et al.*, 1987; Verbalis *et al.*, 1986; Uvnas-Moberg *et al.*, 1985) and discussed elsewhere in this thesis. The findings of the present study in lactating ewes do not, however, contradict the finding that vagotomy inhibits suckling-stimulated OT release in rats (Eriksson *et al.*, 1994). In fact, the present findings further support the concept that feeding stimulates OT secretion in lactating both ruminant and non-ruminant species (Samuelsson *et al.*, 1996b; Svennersten *et al.*, 1995; Svennersten *et al.*, 1990; Uvnas-Moberg *et al.*, 1985). Based on these findings together with the previous results (Chapter 4), it is likely that OT release in response to suckling and also feeding is mediated through the vagus nerves in ewes as well as other species (Samuelsson *et al.*, 1996b; Svennersten *et al.*, 1995; Svennersten *et al.*, 1990; Uvnas-Moberg *et al.*, 1985).

Presentation of food caused a release of insulin in the sham-operated and vagotomized ewes, but the increases were significant only in the sham-operated ewes (Figure 6.3 (b)). This was also reflected in the significantly increased baseline corrected areas under the insulin response curve immediately after presentation of food such as during the periods 1-2 and 2-4 minutes post-feeding, for the sham-operated ewes compared with those of the vagotomized ewes (Figure 6.4). The results confirm, in lactating ewes, the findings of the previous study (Chapter 5) in which vagotomized wethers secreted significantly reduced amounts of insulin in response to presentation of food.

Plasma insulin concentrations rose in response to suckling in the sham-operated ewes, but the concentrations were not affected in the vagotomized ewes (Figure 6.1). Although not statistically significant ($P < 0.10$), the results suggest that vagal nerve activity is an important part of the mechanism(s) regulating suckling-associated insulin release in lactating ewes. These results are in very good agreement with findings from the literature (Franceschini *et al.*, 1990; Eriksson *et al.*, 1987; Widstrom *et al.*, 1984; Uvnas-Moberg *et al.*, 1984; Uvnas-Moberg and Eriksson, 1983) and were discussed in Section 1.4.5.2. These authors suggested that suckling-induced insulin release is probably

secondary to a reflex activation of vagal neurons since the release of insulin is under vagal control. Moreover, the pattern of increase is similar between this and previous studies. For example, in the present study, increased plasma insulin concentrations were maintained for 5 minutes and then started to decline to baseline values. In lactating dogs, suckling induced an immediate three-fold rise in plasma insulin concentration which was maintained for approximately 5 minutes and then started to decline to baseline concentrations (Uvnas-Moberg and Eriksson, 1983). It is possible that sucking the teats causes a reflex activation of the brain stem vagal nuclei such as the DMN of the vagus, which, in turn, through vagal efferents, stimulate insulin release from the pancreas. Since plasma insulin concentrations were apparently not affected by suckling in the vagotomized ewes, it is probable that the neural signals originating from reflexly activated vagal efferents did not reach the pancreatic β-cells due to the interruption of vagal innervation of the GI tract (see Figure 1.3). The efferent pathway from the vagal nuclei to the pancreatic β-cells has been discussed elsewhere in this thesis (Berthoud and Powley, 1990; Berthoud *et al.*, 1990; Kirchgessner and Gershon, 1990,1989; Poulsen *et al.*, 1983).

In conclusion, these results suggest that vagal innervation of the GI tract is important in the CPIR and suckling-associated insulin release in lactating ewes. Further, the finding that CPIR is mediated through the vagus nerves in lactating ewes, is consistent with results from the previous study that the vagus nerves play a major role in the CPIR in wethers. This suggests that vagal-mediated CPIR is a general effect in sheep and perhaps in other ruminant species. Oxytocin results in the present study in general support the findings in other studies in which vagus nerve has been shown to modulate OT release during suckling as well as feeding in several species.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

A large demand for nutrients by the active mammary gland during lactation, particularly in domesticated ruminants bred for dairying production, is met by various strategies that are adapted to act in a well-coordinated fashion during this phase of the reproductive cycle. Although mechanisms regulating nutrient partitioning and milk synthesis are not fully understood in ruminants, clearly greater demand for nutrients by the lactating mammary gland creates diverse changes in whole body metabolism including the metabolism of carbohydrates, protein and fat. In this context, insulin, a key metabolic hormone, plays a crucial role in regulating the supply of substrates to the mammary gland by altering peripheral extramammary metabolism (Metcalf *et al.*, 1991; Tesseraud *et al.*, 1992). A major objective of this study was to investigate the role of the vagal innervation of the GI tract in insulin release in both lactating and wether sheep and also nutrient partitioning and milk production in ewes.

Although insulin secretion is generally thought to be regulated by blood metabolites, the existence of an autonomic neural control mechanism, regulating the rate of secretion of the hormone, was strongly suggested by Woods and Porte (1974). The release of pancreatic insulin in response to intra-jugular injection of glucose was significantly suppressed in vagotomized lactating ewes and wether sheep compared with that of sham-operated controls (Chapters 2 and 5). These results are consistent with the observations of Bloom and Edwards (1981) who showed that atropine, a muscarinic blocker, completely blocked the release of insulin following an intravenous infusion of glucose in calves. Furthermore, pre-treatment of calves with atropine suppressed the rise in plasma insulin concentration in response to electrical stimulation of the vagus nerve.

The results in the present study further indicate that the effect of vagotomy on insulin release during hyperglycaemia is dependent not only on the plane of nutrition but also on the degree of β-cell sensitivity at the time of glucose injection. The presence of food in the GI tract initiates vagal signals and sensitises the β-cells (Chapter 5). In the

present study (Chapter 5), the plane of nutrition and the time of glucose injection relative to time of feeding were expected to alter nutrient availability and hence β-cell sensitivity and their response to glucose injection.

Thus the insulin response to glucose injection was higher in animals fed on the high plane of nutrition than those fed on the low plane of nutrition (Figure 5.2), suggesting that the β-cell response is increased with an increasing nutrient intake. This is consistent with earlier findings (Bassett, 1971), and also with the present findings (Figure 5.4) that postprandial insulin secretion was significantly higher in wethers fed on the high plane of nutrition than those fed on the low plane of nutrition. Further, variations with time after feeding, in the sensitivity of the pancreatic β-cells, as measured by the release of insulin during hyperglycaemia, was clearly demonstrated in the present study with wethers (Chapter 5). In this experiment, wethers were given a bolus injection of glucose 2 h and 22 h after feeding, in which the two periods were considered to represent fed and fasted states, respectively. The insulin response to glucose injection was much greater ($P < 0.0002$) at 2 h than at 22 h after feeding. This indicated that the presence of food in the GI tract or high blood glucose concentrations are important for sensitizing β-cells. However, the findings that the insulin secretion was suppressed after vagotomy of the abomasum, pylorus and duodenum (Figure 5.2), suggests that the presence of food in the GI tract is the predominant factor for the observed effects. These results helped clarify the findings of previous studies with lactating ewes, in which vagotomy suppressed insulin secretion in response to glucose injection when administered 3-4 h after feeding (Chapter 2), but not 6-7 h after feeding (Chapter 4). This suggests that the effect of vagotomy on insulin release in response to glucose injection is more apparent after a short period (i.e. 2-4 h) following feeding than after a longer period (6-22 h). Furthermore, as it was mentioned, it was clear from the results in Chapter 5 that the sensitisation of β-cells is not solely due to blood metabolites in origin. Thus, postprandial insulin secretion is significantly suppressed in vagotomized animals compared with the sham-operated controls, in spite of significantly higher blood glucose levels (Figure 5.4) and, suggest that the stimulus for sensitizing the pancreatic β-cells was vagal in origin. Thus, these results indicate that the pancreatic β-cells are more sensitive soon after feeding, and hence the increased insulin secretion in response to hyperglycaemia, because the vagal inputs reaching the β-cells

from the GI tract are relatively higher due to the presence of food in the GI tract and the amount of food ingested.

The present study (Chapters 5 and 6) also demonstrated that vagotomy suppresses the insulin secretion that normally occurs immediately after the start of eating and before there has been any change in plasma glucose concentration. This cephalic phase insulin release (CPIR), in response to a variety of stimuli has been reported previously in ruminants (Faverdin, 1986b; Porter and Bassett, 1979; Vasilatos and Wangsness, 1980; Chase *et al.*, 1977; Bhattacharya and Alulu, 1975; Bassett, 1974a; Lofgren and Warner, 1972) as well as in other species (Strubbe, 1992; LeBlanc *et al.*, 1991; Berthoud and Powley, 1990; Berthoud and Jeanrenaud, 1982; Teff *et al.*, 1993, 1995). The suppression of CPIR after vagotomy has not been reported previously in a ruminant, however, it is consistent with previous findings in rats (Berthoud and Powley, 1990).

Suckling causes insulin release in several species including human beings (Franceschini *et al.*, 1990; Eriksson *et al.*, 1987; Uvnas-Moberg *et al.*, 1984; Widstrom *et al.*, 1984; Uvnas-Moberg and Eriksson, 1983 and as discussed in Chapter 6). It was proposed that stimulation of the teats causes a reflex activation of vagal nuclei, such as the DMN of the vagus, which, through vagal efferents, stimulates insulin release from the pancreas (Eriksson *et al.*, 1987). In the present study (Chapter 6), suckling caused a greater rise in plasma insulin concentration in the sham-operated control ewes than in the vagotomized ewes (Figure 6.1), and the difference between two groups tended ($P < 0.09$) to be significant. This suggests that the neural signals originating from reflexly activated vagal efferents, did not reach the pancreatic β-cell due to the interruption of vagal innervation of the GI tract in the vagotomized animals. Moreover, these data support the concept that the GI tract and its accessory organs such as the pancreas, brain and mammary gland are all interconnected by means of a neural network (see Figure 1.3). Thus, sensory information carried from the mammary gland (Tindal, 1978; Tindal and Knaggs, 1975) is relayed through the DMN of the vagus to the pancreatic β-cells via efferent vagal fibres (Kirchgessner and Gershon, 1990, 1989; Siaud *et al.*, 1989; Sawchenko *et al.*,1988, 1982; Sims and Lorden, 1986; Kooy *et al.*,1984; Saper *et al.*, 1976). These findings suggest that the vagal innervation of the GI tract is in a

position to moderate the release of insulin associated with suckling, as it does insulin release under several other physiological conditions described elsewhere in this thesis.

It is concluded that the vagus nerve plays a significant role in insulin secretion in response to hyperglycaemia, feeding and the cephalic phase of food ingestion in sheep and that the levels of blood glucose and other metabolites may not play an exclusive role in the regulation of insulin secretion. It is also concluded that the vagus nerve is important in suckling-associated insulin release in this species.

It is well recognised that during lactation milk production is reduced if nutrient intake falls. In addition to a direct effect of nutrient intake on the availability of nutrients to the mammary gland, it has also been proposed that milk production is reduced by a reduced flow of afferent vagal impulses from the GI tract to the brain (Samuelsson *et al.*, 1996b; Uvnas-Moberg, 1989). In support of this, it was suggested that vagotomy interferes with the release of OT and hence milk removal, which in turn leads to a reduction in milk production (Eriksson *et al.*, 1994). In the present study, milk yield in the vagotomized animals fell significantly ($P < 0.05$) on day one after surgery and fat yield was reduced for 2 days ($P < 0.05$) when compared with that of sham-operated controls, but these differences were not associated with differences in intake, or even a fall in intake (Chapter 2). These data are consistent with an effect of vagotomy on milk removal. However, fat and milk yields in the vagotomized ewes were restored within the next 2-3 days and milk yield was not affected by vagotomy in the second experiment with lactating ewes (Chapter 4). It is possible that differences in milk ejection and hence milk removal in these studies may have been partly masked because of the anatomical features of the ewe mammary gland. Thus, in lactating ewes, like goats, adequate milk removal may still occur in the presence of reduced concentrations or even absence of OT because they have large cisterns where milk draining from the alveoli can be stored (Tindal, 1978). This type of anatomical architecture of the mammary gland is in marked contrast to that of rats whose milk drains directly from the lobes to the out side via the ducts. In rats, there are no sinuses or cisterns for the storage of milk so that a small reduction in OT concentration may lead to incomplete milk removal (DeNuccio and Grosvenor, 1971), and inhibition of milk secretion through an autocrine mechanism (Wilde and Peaker, 1990; Wilde *et al.*, 1987, 1988; Henderson and Peaker, 1984, 1987; Henderson *et al.*, 1983). In this

connection, milk yield was significantly decreased with a reduction in plasma OT concentrations in vagotomized rats (Eriksson *et al.*, 1994). Further, it is also worthwhile to note that in the second experiment with lactating ewes (Chapter 4) that OT was injected before each milking. This would have emptied the alveoli and prevented the inhibition of milk ejection in vagotomized animals or expression of any autocrine mechanisms (Henderson and Peaker, 1987) that may have given rise to differences in the yields between the vagotomized and sham-operated animals.

Attempts to demonstrate directly an effect of vagotomy on OT release by measuring OT concentration in the blood gave equivocal results. Baseline OT concentrations were significantly lower in the vagotomized ewes than in the sham-operated ewes (Figure 4.7), but the difference between the two groups in the baseline corrected area under suckling-associated OT response curve was not significantly different, reflecting the large variation in response between animals. In contrast, in the experiment reported in Chapter 6, although OT concentrations were apparently higher in the sham-operated animals than in the vagotomized animals, they were not significantly different. But in this study, simultaneous suckling and feeding did increase ($P < 0.05$) OT concentrations from the baseline values in the sham-operated animals but not in the vagotomized animals (Figure 6.2). Overall, the failure to achieve significant differences consistently in these experiments suggests that a larger number of animals than that used here is needed to overcome the extreme variability of OT concentrations in plasma. However, these data that indicate an effect of vagotomy on OT secretion, are strongly supported by data from the literature (Samuelsson *et al.*, 1996b, 1993; Svennersten *et al.*, 1995, 1992, 1990; Eriksson *et al.*, 1994; Stock and Uvnas-Moberg, 1988; Uvnas-Moberg *et al.*, 1985 and as discussed in Chapters 2 and 4). Furthermore the enhanced feeding-related OT secretion during suckling may be important from dairy management point of view and thus, may be useful in routine milking, particularly in management systems involving automated feeding and milking in dairy cows.

In the literature review it was suggested that vagotomy eliminates the satiety induced by CCK in non-ruminants, which indicates the effect of CCK is mediated through the vagus nerve (Moran *et al.*, 1993; Reidelberger and O'Rourke, 1989; Smith *et al.*, 1981, 1985; Lorenz and Goldman, 1982). Abdominal vagotomy blocks the inhibitory

effect of CCK on food intake in rats and the effect is mediated through the vagal CCK binding receptors (Smith *et al.*, 1985; Shillabeer and Davison, 1985). In light of this, the lack of an effect of vagotomy on intake was unexpected in the present study (Chapter 3), although the vagal CCK binding receptors are present on the ovine vagus nerves (Farningham *et al.*, 1993). Thus, it is not clear from this study whether the vagus nerve is involved in satiety in sheep. However, neuroendocrine pathways by which CCK modulates food intake have yet to be established in ruminants. Further, it is not known whether axons innervating the rumen and reticulum also possess CCK binding receptors. However, further studies involving short term intake responses, as has been widely used in monogastric (Washburn *et al.*, 1994; Covasa and Forbes, 1994; Prete and Scharrer, 1993; Shillabeer and Davison, 1985) and ruminant (Farningham *et al.*, 1993; Farningham and Whyte, 1993; Anil and Forbes, 1988; Martin and Baile, 1972) trials, may provide a better insight into the effect of vagotomy in ruminants.

Although food intake was apparently not affected by vagotomy, digestibility of DM and N was significantly increased in the vagotomized wethers compared to that in sham-operated controls (Chapter 3). The exact mechanism responsible for this increased digestibility in vagotomized wethers is not clear. The major contractions of the forestomach are controlled via the vagus nerves of the GI tract in ruminants (Gregory, 1982; Harding and Leek, 1971; Stavney *et al.*, 1963; Titchen, 1958; Iggo, 1956; Habel, 1956; Duncan, 1953). Therefore, it is unlikely that there was any change in the rate of contraction as innervation of this part of the GI tract was left intact in these experiments. Since the rate of movement of digesta through the GI tract is one of the major factors that determines how much nutrient can be obtained by an individual animal from a given diet, it is possible that the increased digestibility following vagotomy might have resulted from a decreased abomasal emptying leading to an increased food retention time.

There are several ways by which vagotomy might slow abomasal emptying in sheep. It is possible that the rate of digesta flow may have decreased due to the interruption of vagal sensory nerves innervating the abomasum and duodenum, and which reflexly affect reticulo-rumen motility (Titchen, 1968). Alternatively, SS, a hormone known to be secreted from the abomasum and small intestine as well as the pancreas in sheep and dogs (Reddy *et al.*, 1984; Schusdziarra *et al.*, 1978, 1979), may mediate a

reduction of abomasal emptying in the vagotomized animals (Barry *et al.*, 1982). Although plasma SS concentrations were not measured in the present experiments, SS inhibits the passage of digesta through the abomasum as well as small intestine in sheep (Faichney and Barry, 1984) and gastric emptying in dogs (Bloom *et al.*, 1975). Thus, severance of the vagal innervation to the GI tract may be expected to reduce gastric emptying through an increased SS secretion, leading to an increased digestibility of food. Indeed, severance of the gastric vagal innervation caused an increased release of SS in lactating rats (Eriksson *et al.*, 1994). Vagal stimulation inhibited the release of SS in cats (Uvnas-Wallensten *et al.*, 1980) and pigs (Holst *et al.*, 1992, 1981). Furthermore, atropine treatment strongly inhibited gastric emptying in calves (Bloom *et al.*, 1978). Thus there are strong data indicating that SS secretion is under the control of the vagal innervation to the stomach and small intestine (Section 1.4.3.2). Thus one explanation for the increased digestibility of DM and N observed in the present study is a slower rate of passage in the vagotomized animals mediated by an increase in SS secretion.

The various responses measured in both the abomasal, pyloric and duodenal vagotomized (ADV) and hepatic, abomasal, pyloric and duodenal vagotomized (HADV) groups were all similar in magnitude (Chapter 5), which indicates that HADV did not have any additive effect over that achieved by the ADV alone. Therefore, the findings indicate that the hepatic branch is not implicated in modulating insulin release in sheep. Thus, it is concluded that the vagal fibres contained in the abomasal, pyloric and duodenal branches of the vagus nerves are solely responsible for eliciting the insulin responses during hyperglycaemia, feeding and feeding related cephalic phase in wethers.

Conclusions

Finally, it is important to consider the physiological significance of these studies, which may be summarized as follows;

1. The results from these studies (Chapters 2, 4, 5, and 6) strongly suggest that insulin secretion is modulated by the vagal innervation of the GI tract in sheep. It is concluded that afferent vagal neural signals from the GI tract and efferent signals from the vagal nuclei in the brain stem are important in sensitizing the

pancreatic β-cells and in eliciting insulin release in response to a variety of stimuli such as hyperglycaemia, feeding and the cephalic phase of feeding.

2. The influence of the vagus nerves on insulin secretion was further confirmed in acute experiments, in which peripheral cut ends of the cervical vagus nerves were electrically stimulated using standard physiological procedures (Chapters 4 and 5). Insulin secretion in response to electrical stimulation was suppressed in the vagotomized animals.

3. The results indicated that, as in other species, insulin release in response to suckling is a vagally mediated phenomenon in sheep. However, because the differences were not statistically significant, further investigation is required to confirm this observation in a ruminant species.

4. The vagus nerve influences OT secretion in response to suckling as well as under basal conditions in ewes (Chapters 4 and 6). Simultaneous suckling during feeding enhanced OT secretion in the sham-operated animals but not in the vagotomized ewes, suggesting that the vagus nerve is also involved in the feeding-associated OT release in lactating ewes. This may be important from dairy management point of view and thus, may be useful in routine milking, particularly in management systems involving automated feeding and milking in dairy cows.

5. The present studies with lactating ewes (Chapters 2 and 4) failed to demonstrate a consistent effect of vagal innervation of the GI tract on milk yield and composition. However, because of the particular anatomy of the mammary gland of the ewe, the effect of vagotomy on OT concentration may not have affected the yield.

6. The vagal innervation to the abomasum, pylorus and duodenum is important in the regulation of nutrient availability for absorption in sheep, as reflected from the results of the digestibility trials (Chapter 3). A definite role for the vagus nerve in the regulation of digestibility remains to be established. This requires a further investigation in vagotomized sheep in which the release of SS and the

rate of movement of digesta through the abomasum are investigated. Nevertheless, the vagal involvement in the control of food intake requires further investigation especially the neuroendocrine pathways by which CCK modulates food intake.

8. Further experiments are necessary to investigate the role of the vagus nerves in the release of plasma hormones such as GH, PRL, SS and gastrin in a ruminant in which diurnal patterns of the hormone concentrations should be measured in order to minimise any effect of the time after feeding.

APPENDIX 1

RADIOIMMUNOASSAY OF OVINE SERUM/PLASMA OXYTOCIN

INTRODUCTION

It was necessary to develop and validate an OT assay since the laboratory did not have one. The measurement of OT in plasma is difficult for several reasons. First, the plasma concentrations of the hormone are generally very low in cows and sheep (Svennersten *et al.*, 1990; Fuchs *et al.*, 1987). Secondly, the amount of OT release under physiological conditions, such as during suckling (Eriksson *et al.*, 1994; Higuchi *et al.*, 1983; Fuchs *et al.*, 1987), milking (Samuelsson *et al.*, 1996), feeding and milking (Svennersten *et al.*, 1995; Svennersten *et al.*, 1990), and the oestrous cycle (Moore *et al.*, 1986; Schams *et al.*, 1982), is also small. Thirdly, the presence of binding proteins in plasma or serum and the non-specific precipitation of these proteins with OT antibody interfere to give incorrect hormone concentrations.

Because of these plasma protein interferences, it is necessary to extract and concentrate the plasma/serum OT before assay, a requirement that significantly limits the speed and efficiency of the OT-RIA. Methods used to extract OT in plasma/serum include the use of Sep-Pak-Plus C_{18} cartridges (Kjoer *et al.*, 1995), Fuller's earth (Dawood *et al.*, 1978), or acetone/ether extraction (Robertson *et al.*, 1973; Schams *et al.*, 1979). Sep-Pak-Plus C_{18} cartridges are widely used for plasma OT extraction (Kjoer *et al.*, 1995; Eriksson and Uvnas-Moberg, 1990; Seckle and Lightman, 1988; Stock and Uvnas Moberg, 1985, 1988; Schams, 1983) and give consistently satisfactory recoveries of plasma OT and require low plasma volumes compared to other methods. Thus extraction with Sep-Pak-Plus C_{18} cartridges was the preferred method of extraction in the present study. The method used was based on that described by Kjoer *et al.* (1995) and is described in detail below, together with a description of the RIA procedures.

MATERIALS AND METHODS

Source of hormone and reagents

Synthetic OT (Bachem, H-2510, Lot No. A22372; Sigma Chemical Co., St. Louis, Mo 63178 U.S.A., Lot No. 125H5810) was used for the reference standards as well as for iodination with ^{125}I as the radioactive trace. $Na^{125}I$ was obtained from Amersham International plc, Amersham, UK; Sephadex G-25 (fine, Lot No. 230841) from

Pharmacia Biotech, Uppsala, Sweden; P-2 (Bio-Gel) from Bio Rad Laboratories, Pvt. Ltd., New Zealand. Bovine serum albumin (BSA, Fraction V, Lot No.12887426-55) was obtained from Boehringer, Mannheim GmbH, W. Germany. Antibody to OT raised in rabbits (Robinson 1115 made in 1980) was kindly donated by Dr I. C. A. F. Robinson. Activated charcoal (Lot No. 92F-0304) and polyethylene glycol (PEG 8000, Lot No. 74H0407) were obtained from Sigma Chemical Co., USA.

Preparation of standards

The assay buffer used for preparation of reference standards, as well as for OT assays was 0.1M Tris (pH 7.3) in 0.3% BSA and 0.01% NaN_3 at pH 7.3. Standards were prepared from a stock solution containing 10 µg/ml OT (Cahn Elctrobalance, Model 4600, Cahn instruments, Paramount, California 90723, USA, was used to weigh OT) in 0.01M acetic acid. Standards containing 2560, 1280, 640, 320, 160, 80, 40, 20, 10, and 5 pg/ml were prepared by serial doubling dilutions with assay buffer commencing with a solution containing 5120 pg/ml. Standards were aliquoted into 1 ml lots and stored frozen at -20^0 C for up to 6 months until used in the assays.

Reference samples

Five quality control reference samples were made up with pooled plasma collected from non-lactating well fed sheep. One of five samples was labelled as sample 1 which was not spiked whereas samples 2, 3, 4, and 5 were spiked using synthetic OT and made 20, 50, 100, and 200 pg OT/ml, respectively. These reference samples were extracted and stored frozen.

Preparation of ^{125}I-labelled oxytocin

Radioiodination

The iodination of OT was based on the chloramine-T method originally described by Greenwood *et al.* (1963) and as modified by Moore *et al.*, 1986 (and personal communication).

Five µl (0.5 mCi) of $Na^{125}I$ was placed in a siliconized V-bottom iodination vial, followed by 20 µl of 0.5M phosphate buffer (0.5M PB, pH 7.4). 5 µl of OT from a solution containing 1 µg/µl OT (in 0.01M acetic acid) was then added to iodination vial

and mixed using a siliconized pasteur pipette. 20 µl of freshly prepared 0.34% chloramine-T in 0.5M PB, pH 7.4, was added to the vial rapidly and mixed well. Sixty seconds later the reaction of chloramine-T induced oxidation was stopped by rapid addition of 200 µl of 10% bovine serum albumin in 0.1M Tris buffer (pH 7.3).

Purification

Purification of iodinated OT (^{125}I-OT) was carried out using Sephadex G-25 (fine) column. A 1x10 cm column of Sephadex G-25 was prepared with 2g of Sephadex G-25 in approximately 50 ml of 0.1M Tris buffer (pH 7.3) by allowing it to swell overnight at 4^0 C. The slowly settled column (with total gel bed volume of about 10 ml) on a glass bead and glass wool plug was then equilibrated and conditioned with 0.5 ml of 10% BSA and eluted with 15 ml of 0.3% assay buffer.

The contents of the iodination vial were transferred to Sephadex G-25 column and eluted with assay buffer and 0.5 ml fractions were collected into thirty LP3 polystyrene assay tubes (Luckhams Co., England). 10 µl from each fraction were counted for 60 seconds for plotting the elution profile on a gamma counter (LKB Wallac, 1261 Multigamma, Wallac OY, Finland). The fractions with the highest counts were further purified.

Further purification of ^{125}I-labelled oxytocin on P-2 (Bio Gel)

A 1x10 cm column of P-2 was prepared with 3g of P-2 in approximately 30 ml of Tris buffer (pH 7.3) by allowing the resin to swell overnight at 4^0 C (Total gel bed volume was about 10 ml). The slowly settled column on a glass bead and glass wool plug was then equilibrated with 0.5 ml of 10% bovine serum albumin and eluted with 15 ml of 0.3% assay buffer. Samples (0.25 ml) from the Sephadex G-25 column were loaded onto the P-2 column and eluted with assay buffer. A total of 30 fractions was collected and 10 µl from each fraction were counted for 60 seconds in the gamma counter and the elution profile plotted.

Test for maximum trace binding

The fractions with the highest counts from the P-2 column were tested to identify the trace that gave the highest binding percentage in the OT assay. Three tubes for trace

(Total count tubes-TCT) and six B0 (Binding at zero standard or sample) tubes were included for each fraction tested. 100 µl of OT-antibody (diluted 1: 40,000 in assay buffer) was added to the B0 tubes. 50 µl of trace containing approximately 5000 CPM was added to TCT and B0 tubes. Tubes were well mixed using a vortex mixer (Thermolyne, Maxi-Mix III type, 65800) and incubated for 24 h at 4^0C. After incubation 2 ml of a solution containing charcoal stripped ovine plasma (CSOP; prepared as described below) and 20% polyethylene glycol (20% PEG) was added to B0 tubes, mixed and left at room temperature for 1 h. They were then centrifuged at 3500 rpm for 15 minutes at 4^0C and the supernatant was decanted carefully. Precipitates were counted in a gamma counter and plotted for binding percentages (i.e. mean B0's / TCT x 100). The samples of trace that gave the highest percentage binding, generally 40 to 60%, were used in the assays of the unknown samples collected from the experiments.

Charcoal-stripped ovine plasma

Sheep plasma samples were stirred gently overnight with activated charcoal (1 g of charcoal per 100 ml plasma) at 4^0 C and then centrifuged and filtered through whatman No:1 filter papers. The resulting charcoal-stripped ovine plasma (CSOP) was aliquoted and stored frozen until used in the assays. CSOP together with 20% polyethylene glycol (20% PEG) prepared in 0.01M PBS, pH 7.5 (Desbuquois and Aurbach, 1971) was used to facilitate the separation of antibody-antigen complex from unbound trace in the assay.

Serum/plasma extraction procedure

Sep-Pak-Plus C18 cartridges (Millipore Corporation, Milford, MASS, USA, Lot No. T5231G1) were primed by the passage of 5 ml of a mixture of 89% Methanol (89Meth) and 6% acetic acid (6AA) in a ratio of 89:11 followed by three 5 ml aliquots of 6AA.). Thawed serum/plasma sample (500 µl or 1.0 ml) was transferred to a new vial and 50 µl of 1N HCI added to acidify to pH 2-3. This was followed by passing 5 ml 6AA 3 times through the cartridge. 3 ml of 6AA was then added to the already acidified serum sample, poured into a syringe and gently pushed through the cartridge. The sample vial was rinsed with 3 ml 6AA which was then passed through the cartridge. Serum/plasma proteins were then washed through the column with two 5 ml aliquots of 6AA two times. Oxytocin was then eluted from the cartridges with 3ml of the 89:11 mixture of 89Meth

and 6AA and the elutes were collected into polypropylene tubes. Oxytocin was first eluted with 3 ml of the mixture and 3 minutes later OT was eluted again with a further 1.5 ml of the mixture. C_{18} cartridges were cleaned by washing them with 5 ml 89Meth twice, followed by three washes with 5 ml distilled water and reused up to 10 times. Extracts were airdried (BT3, Grant Instruments (Cambridge) Ltd, Barrington, Cambridge, CB25QZ, England) overnight at 37.5^0 C and stored frozen until assayed. They were reconstituted with 1 ml of assay buffer before used in the assay.

Extraction recovery studies

The recovery of OT using the extraction procedure described above was tested using standards in assay buffer or cow/sheep sera. Known quantities of OT from the OT stock solution were added to pooled cow or sheep serum (pooled sera were tested for OT concentrations and were taken into account when calculations were done) to achieve final concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, and 5120 pg/ml above that already present in the pooled sera. Alternatively serum samples containing a range of OT concentrations were obtained by preparing a serum sample with 5120 pg OT/ml and then performing a series of doubling dilutions with serum to achieve final concentrations from 5 to 5120 pg/ml. 0.5 ml or 1 ml of samples of standards or standards in pooled sera was extracted, air dried and stored frozen. They were reconstituted in 1 ml assay buffer before assay.

Antibody dilution study

An assay was carried out to determine the optimum dilution of the OT antibody. Three dilutions of antibody, 1:40,000, 1:50,000, and 1:60,000, in assay buffer were used. The assay included a complete set of standards, TCT, NSB, and B0 tubes for each dilution of antibody tested.

RIA method

The procedure for the OT RIA was as follows. The assay included TCT (Total count tubes), NSB (non-specific binding), BO tubes (binding at zero standard/sample), standards, 5 reference samples and unknown samples. Oxytocin standards (100 µl) or extracted reference samples or extracted unknown serum/plasma samples were added to 5

ml polystyrene assay tubes (Cat. No. 55.483, Sarstedt, Sarstedt Australia Pvt Ltd., Ingle Farm, Australia) and mixed with 100 µl of OT antibody (diluted at 1:40,000 in assay buffer) using a vortex mixer. Assay buffer (100 µl) instead of standard/sample, was added to B0 tubes together with OT antiserum. Assay buffer (200 µl), instead of standard/sample + antibody, was added to NSB tubes. After 48 h incubation at 4^0 C, 50 µl trace (^{125}I-OT in assay buffer) containing approximately 5000 cpm, was added to every tube including TCT, mixed, and incubated for 24 h at 4^0 C. On day 4 following incubation, 2 ml of a solution containing CSOP/20%PEG (CSOP, 20%PEG, and distilled water were at 1.23, 74.07, and 24.7%, respectively) cooled to 4^0 C, was added, mixed well using a vortex mixer and incubated at room temperature for 1 h. Following incubation, tubes except TCT were centrifuged at 3500 rpm for 15 minutes at 4^0 C. The supernatant was carefully removed by aspiration. Radioactivity in the precipitate was counted in the gamma counter for 60 seconds. The value of B0 varied from 20 - 60% of total counts (B0-NSB/TCT x 100), and the value of NSB from 1.1 to 2.3% of total counts for the assays.

Parallelism

The presence of parallelism is an essential prerequisite when developing a valid RIA. The ability to displace labelled antigen from binding to its antibody by the unknown antigen (i.e. hormone in the unknown samples) is termed parallelism and the degree of such displacement is usually compared with the standards. To evaluate this, 50, 100, and 150 µl of six extracted and five unextracted samples and each four of extracted and unextracted reference samples were assayed in triplicates.

RESULTS

Validation of the RIA for oxytocin

Oxytocin radioiodination and purification

Two radioactive peaks were eluted from the Sephadex G-25 (fine) column (Fig. A1). The first peak was occurred between fractions 5 and 7 which corresponds to the void volume of the column and consequently contains compounds with a molecular weight in excess of 5000 Dalton. The second peak of radioactivity was seen between fractions 12

and 16 with the highest radioactivity counts usually found in fractions 13, 14, and 15. These fractions would be expected to contain compounds with a molecular weight of less than 1200 and would include ^{125}I bound to OT and free ^{125}I.

Figure A1 shows that the binding of trace to the OT antibody increases through the profile with the highest percentage of binding was achieved in the fractions on the right side of the second peak. However, because the maximum total binding obtained was only 18% it was necessary to further purify them in order to get reasonably high percentage of total binding. Despite the fact some authors (Dawood *et al.*, 1978; Blank and DeBias, 1977; Chard *et al.*, 1970) have used Sephadex G-25 and that is adequate for separating polymers of OT from dimer it is not adequate to separate the dimer from the monomer and free iodine since Sephadex G-25 separates molecules of molecular weights in the range of 1000 to 5000 Dalton (Manufacturer's instruction, Pharmacia Biotech, Uppsala, Sweden). Therefore, it was necessary to use P-2 (Bio-Gel) column for further purification.

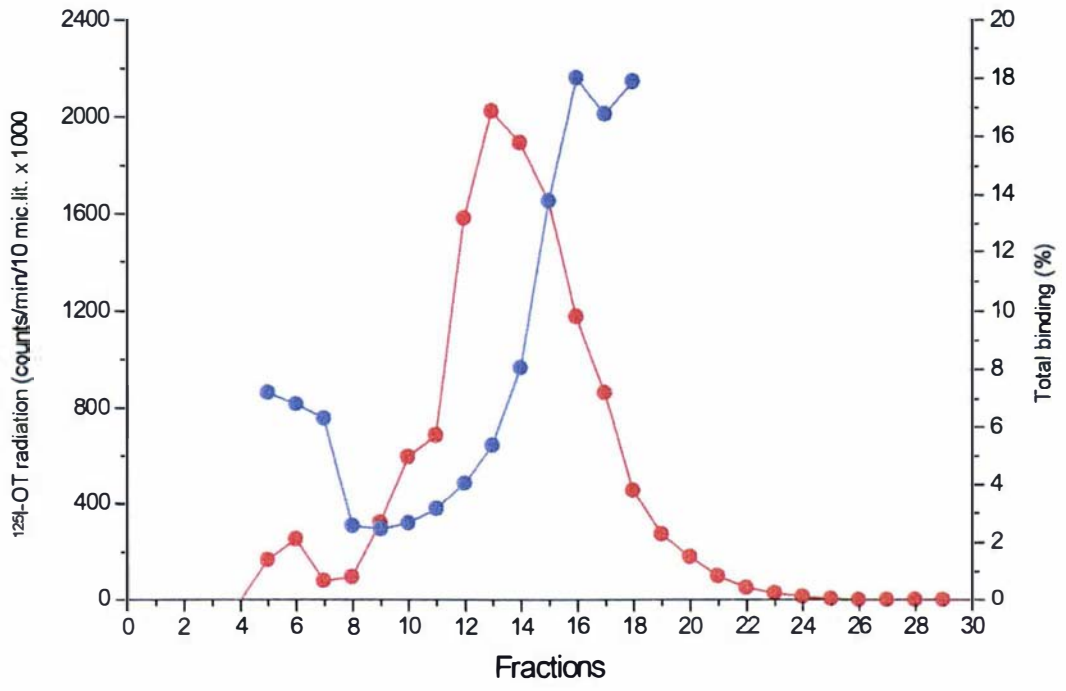

Figure A1 The elution profile of ^{125}I-OT (●—●) and percentage of ^{125}I-OT bound (●—●), purified by Sephadex G-25 (fine) column equilibrated with assay buffer (0.3% BSA in 0.1M Tris buffer). (each point = mean of 6 tubes).

The elution profile from a P-2 column for a fraction from the G25 column is shown in Figure A2, together with the elution profile of free ^{125}I. There was a small peak of radioactivity in fractions 6-7 corresponding to the void volume, but most of the radioactivity was associated with the second peak. A 1x10 cm long P-2 column (with 3 g of gel) contains approximately 10 ml of total gel bed volume, corresponding to a void volume of 3-3.5 ml. The molecules with a molecular weight more than 1800 Dalton are, therefore, expected to be eluted in the void volume. Since the P-2 gel separates the molecules with a molecular weight between 100-1800 Dalton (Manufacturer's instructions, BIO RAD Laboratories Pty Ltd., New Zealand), the use of P-2 column resulted in a better fractionation of ^{125}I-OT monomer and dimer from free iodine.

Figure A2 shows that the percentage bound was low in fractions in the first peak and gradually increased in fractions in the second peak. Fractions containing considerable counts together with a higher percentage of trace bound from the P-2 column, such as the fractions 12 to 14, were selected to use as trace in later assays.

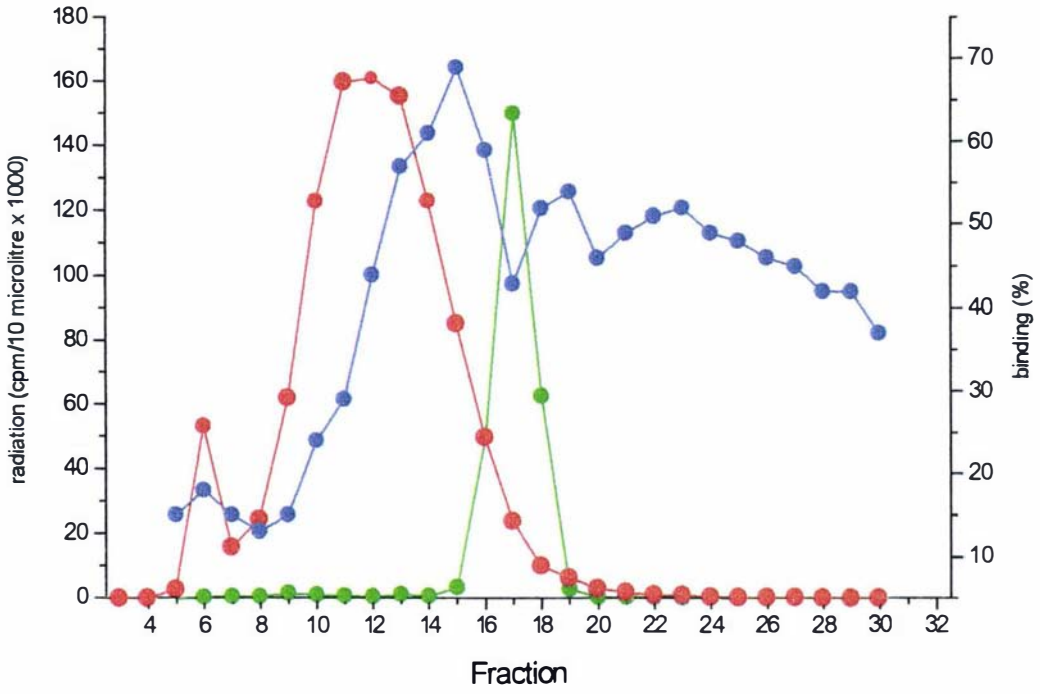

Figure A2 The elution profile of ^{125}I-OT (●—●) from a P-2 column (equilibrated and eluted with 0.3% assay buffer) for a fraction from the G25 column and percentage of ^{125}I-OT bound (●—●) (each point = mean of 3 tubes). ^{125}I elution profile,●—●.

Antibody dilution

Out of three dilutions of antibody tested 1:40,000 resulted in the highest assay binding at zero standard/sample while the rest of the dilutions resulted with a comparatively low assay binding at zero standard/sample (Fig. A3). The 1:40,000 dilution of antibody was, therefore, considered satisfactory and used in all subsequent assays.

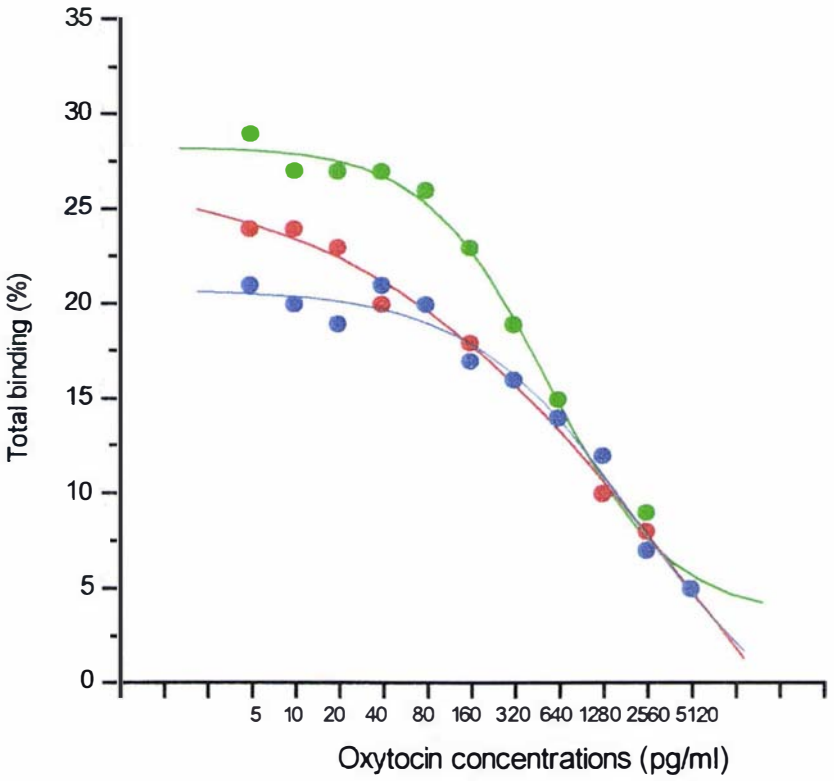

Figure A3 Antibody dilutions. Standard curves for absolute binding of antibody diluted at 1:40,000 (●—●), 1:50,000 (●—●), and 1:60,000 (●—●).

Parallelism

The parallelism of OT RIA was assessed with OT antibody at 1:40,000 dilution in an assay in which 50, 100, and 150 μl of unextracted and extracted samples, as well as extracted and unextracted reference samples, were assayed in triplicate. The lack of parallelism was confirmed if there were consistent deviations between the results of different dilutions. Figure A4 shows binding to OT-antibody by the OT in the unknown samples in 3 different dilutions of each sample that the degree of displacement of labelled-OT was parallel to the OT standard curves.

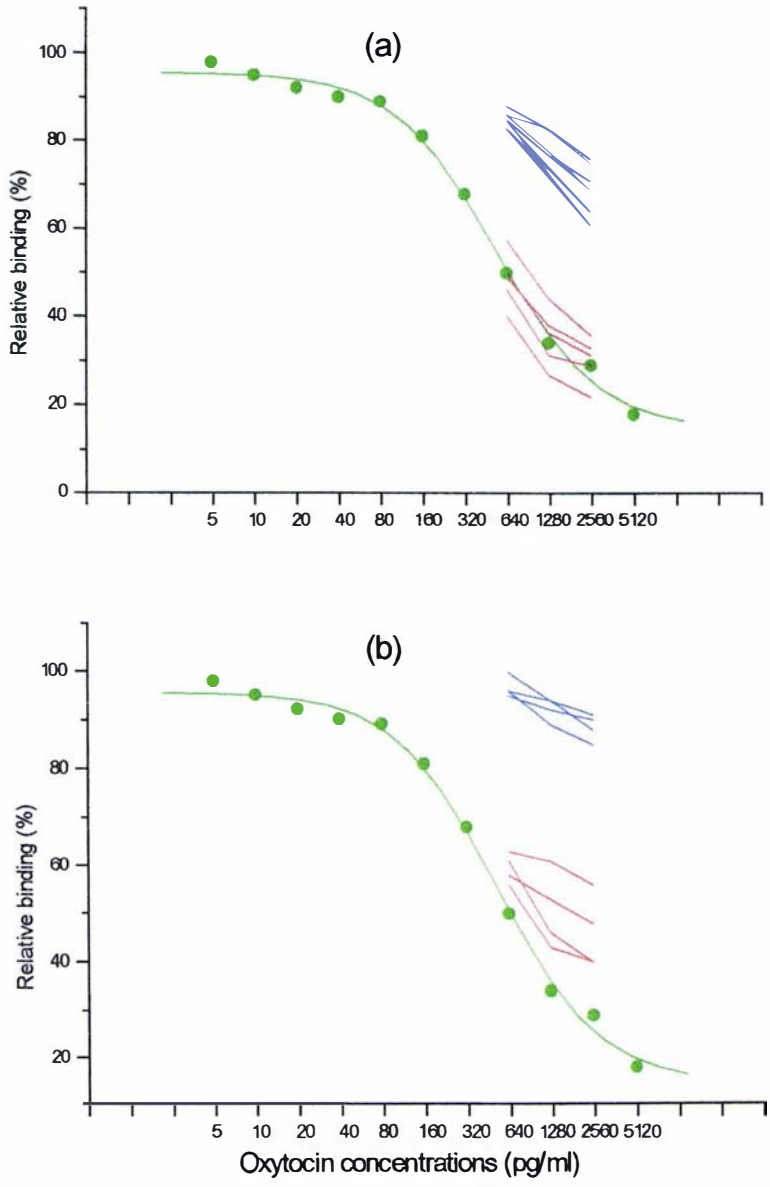

Figure A4 Evidence of parallelism: Standard curves of oxytocin (●—●) were produced with oxytocin antibody at 1:40,000 dilution. The displacement of ^{125}I-OT by oxytocin in extracted (—) and unextracted (—) samples is shown (normal serum samples (a) and reference samples (b)) when they were added at three different dilutions (i.e. 50, 100, and 150µl). (Absolute binding in the assay was 22%).

Serum extraction studies

The mean (±SEM) recovery percentage of OT standards in assay buffer was 100 ± 3 (n=8). Dilutions of extracted standards in assay buffer produced inhibition of label (^{125}I-OT) binding to OT antibody that was parallel to the inhibition produced by dilutions of standard hormone (unextracted standards) in assay buffer (Fig. A5).

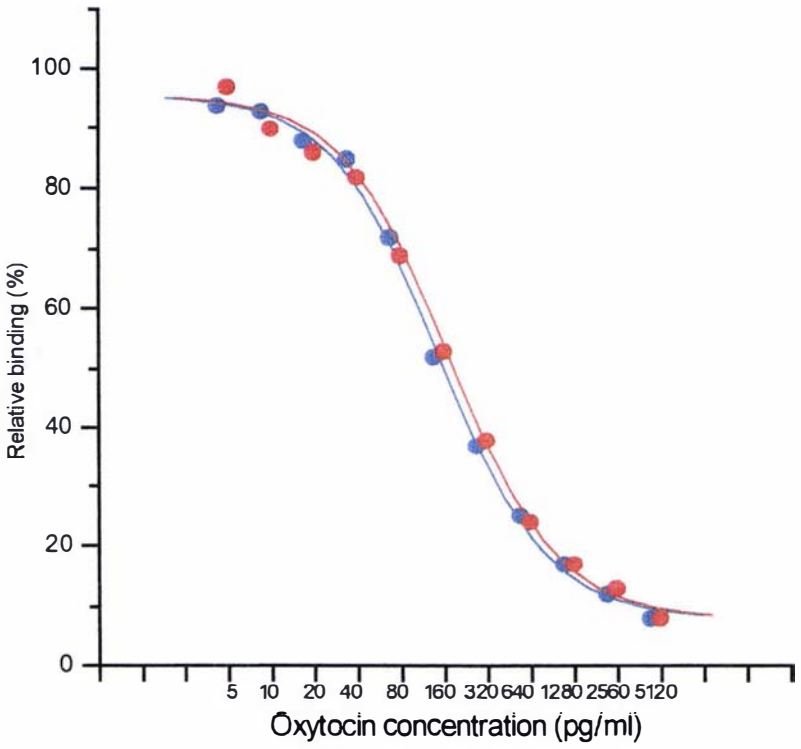

Figure A5 Oxytocin recovery studies. Oxytocin standard curve with relative binding percentage against oxytocin concentration (●—●), and recovery of synthetic oxytocin in standards (in 0.3% assay buffer) after extraction (●—●). Each point on the extract curve represents the mean percentage relative binding produced by three tubes with each 100µl of extracts (reconstituted in 1.0 ml assay buffer) (Absolute binding in the assay was 35%).

The mean percentage (±SEM) recovery of OT from a pooled sheep serum was 82 ± 7 (n=11). The extraction recovery percentages for varying concentrations of OT in pooled serum are given in Table A1.

Table A1. Recovery of added oxytocin in pooled sheep serum. Mean recovery from 5 to 5120 pg/ml (n=8) was 82 ± 7 (±SEM).

Oxytocin added (pg/ml)	Mean recovery percentage (±SEM)	Number of assays
5	70 ± 11	2
10	88 ± 4	2
20	61 ± 4	2
40	78 ± 3	2
80	111 ± 3	3
160	77 ± 5	3
320	73 ± 3	3
640	91 ± 6	2
1280	89 ± 5	3
2560	90 ± 14	2
5120	73 ± 2	2

Sensitivity

The sensitivity of an OT assay is defined to be as the smallest amount of unlabelled OT in a standard curve which can be detected as statistically significantly ($P < 0.05$) different from a zero standard/sample in triplicate determination of the standard curve. Over five assays the mean (±SEM) sensitivity of the RIA was 5.99 ± 2.24 pg OT/ml serum.

Precision (reproducibility)

Precision is a measure of the variation observed between repeated determinations on the same sample. Intra-assay precision was determined by calculating the mean coefficient of variation (CV%) between triplicate determinations of unknown samples. The inter-assay coefficient of variation was calculated on the basis of mean values of triplicates for five control reference samples (with serum OT concentrations spiked from 20 up to 200 pg/ml) analyzed in 5 assays. The intra-assay coefficient of variation (±SEM) ranged from 9.2 ± 0.9 (n=40) to 10.1 ± 0.6 (n=96). The inter-assay coefficient of variation (±SEM) was 14.1 ± 1.0 (n=5).

DISCUSSION

The first ^{125}I peak from the Sephadex G-25 (fine) column at fractions 5, 6, and 7 shows that the molecules with high molecular weight are eluting in the void volume. The radioactivity of this peak may also include aggregated, damage hormone together with protein-bound ^{125}I-labelled OT (Oyama *et al.*, 1971). This also includes labelled albumin because the reaction is stopped by adding bovine serum albumin. A majority of immunoreactive OT was found later in the second peak. However, absolute binding was low because a large proportion of the radioactivity in the fraction was not associated with immunoreactive OT due to a low percentage of labelled OT in fractions from the G25 column. The use of a P-2 column for further purification of these fractions resulted in the production of satisfactorily high percentage of labelled OT.

The composition of the radioactive material in the second peak from both the G25 and P-2 column is apparently not homogenous (Greenwood *et al.*, 1963). This could be due to labelled OT molecules that have bound more than one iodine molecule, giving high radioactivity but low binding in the early part of the second peak. The chloramine-T oxidises the iodide to iodine which then displaces the OH group on the tyrosine molecule. It could contain a mixture of OT dimer with 1 to 4 iodines per OT aggregate, OT monomer with one or more iodine per molecule or even OT fragments, reflecting a high radioactivity with low binding. Thus, the OT monomer with one iodine molecule and the highest binding would be expected in the fractions in the later part of the second peak.

Free iodide would be expected to account for much of the radioactivity that did not bind (about 40 to 60%) to the antibody in these fractions. Non-homogenous distribution of immunoreactive material along the chromatography gel, similar to OT, has also been described for the other neurosecretory small peptide, arginine-vasopressin (Roth *et al.*, 1966). Although it may be possible to reduce the content of free iodine by increasing the column length (Blank and DeBias, 1977; Chard *et al.*, 1970), it was not considered necessary since the main objective of the use of P-2 column was to select a better fraction that could be used in the assay.

Serum extraction is one of the most important steps in the RIA of small peptides like OT (Burhol, 1984 ; Kjoer *et al.* 1995). The assay interference by various serum proteins hinders the accuracy of assay giving misleading values (for example, see Figure A4 for differences in relative binding percentages between unextracted and extracted samples). Because of this, use of a serum extraction step is essential before samples are analysed for OT in the RIA. We initially used an acetone/ether extraction procedure to study OT recovery (Robertson *et al.*, 1973; Schams *et al.*, 1979) but recoveries from this extraction method were very variable and therefore, it was not selected for further use. However, recoveries were consistent using Sep-Pak-Plus C_{18} cartridge. The recovery of OT in serum samples extracted by the Sep-Pak-Plus C_{18} cartridge method was 82 ± 7 (±SEM) which is in good agreement with the results of others (Kjoer *et al.*, 1995; Seckle and Lightman, 1988; Schams, 1983). This was considered satisfactory, as a recovery rate of 82 ± 7% would recover enough hormone in samples with low hormone concentrations. Further, the extraction recovery rate reported here is in good agreement with the results of others (Kjoer *et al.*, 1995; Seckle and Lightman, 1988; Schams, 1983).

Out of three different dilutions for OT antibody tested, 1:40,000 antibody dilution yielded a better standard curve and the highest binding whereas the standard curves from the rest of the dilutions were not as satisfactory. One in 40,000 OT antibody dilution was, therefore, selected and used in all assays.

Parallelism, the ability to displace labelled antigen from binding to its antibody, was well recognised in this study. The results in Figure A4 show that there were parallel changes between the curves of OT standards and samples of both extracted and

unextracted serum/plasma. A lack of parallelism can indicate that natural hormone in the samples differs from the hormone used in the standard curve in molecular structure or immunoreactivity, the binding between the hormone in the samples and the antibody is interfered with by other factors such as some serum proteins. The degradation of radio-labelled hormone during incubation can also result in a lack of parallelism. Further, the results indicate that extraction procedure used improved the parallelism.

Basal as well as suckling-stimulated plasma OT concentrations were reported to be low in lactating ewes (Fuchs *et al.*, 1987) and so an assay with high sensitivity was required for the current studies. The sensitivity of the assays was in good agreement with the results of others (Schams, 1983; Blank and DeBias, 1977; Dawood *et al.*, 1978) and permitted the measurement of low concentrations of OT in serum/plasma samples collected from experiments in lactating ewes. Intra- and inter-assay coefficients of variation (CV%) calculated from unknown samples and reference samples, respectively, were within acceptable limits (Schams, 1983; Dawood *et al.*, 1978 ; Schams *et al.*, 1979; Higuchi *et al.*, 1986, 1985).

In conclusion, serum/plasma extraction, radioiodination, separation of hormone-bound label, and assay procedure described here fulfil the criteria of a RIA method that can be used to measure ovine serum/plasma OT with high assay precision and assay sensitivity. The release of OT during suckling and/or with simultaneous feeding in lactating ewes is discussed in Chapters 4 and 6.

APPENDIX 2

PUBLICATIONS

The following publication arose directly from this thesis.

Herath, C. B., G. W. Reynolds, D. D. S. Mackenzie, S. R. Davis and P. M. Harris. (1996). The role of the vagal innervation of the gut in insulin release in lactating ewes. *Proceedings of the New Zealand Society of Animal Production.* 56: 110-113.

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