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FEED INTAKE REGULATION - ROLE OF CHOLECYSTOKININ IN RUMINANTS

by

ANNETTE H. SUOMINEN ©

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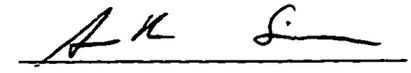

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled FEED INTAKE REGULATION - ROLE OF CHOLECYSTOKININ IN RUMINANTS by ANNETTE H. SUOMINEN in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in NUTRITION AND METABOLISM.

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Dated, Edmonton, September 26th, 1997

"When we try to pick out

anything by itself,

We find it hitched to

everything else in the universe."

John Muir

Dedication

To my husband Cam for all his love, support, and patience

To my parents for raising me to be the person I am

Abstract

The release of the gastrointestinal hormone and neurotransmitter cholecystokinin (CCK) in response to nutrients in dairy cattle was investigated in studies designed to determine the role of CCK in the control of feed intake in ruminants.

In the first experiment the effects of feed restriction and physiological state on CCK gene expression and CCK release were examined. Only a low abundance of CCK mRNA was detected in duodenal tissue collected postmortem. No differences in abundance of CCK mRNA were observed between fasted lactating, fed lactating, or fed non-lactating dairy cows. However, plasma CCK concentration were found to be reduced in animals fasted for three days prior to slaughter.

In order to overcome the disadvantages associated with using postmortem tissue we developed an in vivo intestinal biopsy technique using a fiberoptic endoscope, which was used for the remaining experiments. In those experiments the changes in CCK, but also changes in proglucagon mRNA abundance (encoding the gastrointestinal hormone, glucagon-like peptide-1 (GLP-1)) were evaluated.

In the second experiment, five intestinally-cannulated heifers were feed restricted and subsequently refed. Blood samples and duodenal and ileal biopsies were collected prior to and during feed restriction, and during refeeding. Cholecystokinin and GLP-1 plasma concentrations and CCK mRNA abundance decreased during fasting and increased during the refeeding period; all values returned to their pre-fasting levels at 36, 8, and 16 h after refeeding, respectively. Proglucagon mRNA abundance did not change during fasting or refeeding. The heifers' feed intake throughout the first 10 h after

feeding did not differ before or after fasting but the 24 h feed intake on the first day of refeeding was depressed to 70% of pre-fasting feed intake. The feed intake on the second day of refeeding was not different from pre-fasting intake.

The effects of abomasal infusion of saline, soybean trypsin inhibitor, phenylalanine, oil, casein, or starch and duodenal infusion of trypsin on CCK and GLP-1 release as well as on CCK and proglucagon mRNA abundance were investigated in the last study using two 4 x 4 Latin square design experiments. Blood samples and duodenal and ileal biopsies were taken prior to, throughout, and after the 4 hour infusions. The treatments had no detectable influence on CCK or GLP-1 plasma concentration, CCK or proglucagon mRNA abundance, or feed intake. The results do not support the hypothesis that CCK is involved in short-term control of intake associated with meal feeding, however fasting and refeeding resulted in significant changes in plasma CCK and CCK mRNA.

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Feed Intake Regulation
- Role of Cholecystokinin in Ruminants

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LIST OF ABBREVIATIONS

bp =	base pair
CCK =	cholecystokinin
°C =	degree Celsius
cDNA =	complementary deoxynucleic acid
CMC =	carboxymethyl cellulose
cpm =	counts per minute
dpm =	desintegrations per minute
EDTA =	ethylene-diaminitetetraacetate
GIT-CsCl =	guanidine isothiocyanate - caesium chloride
GLP-1 =	glucagon-like peptide-1
GLP-2 =	glucagon-like peptide-2
LiCl =	lithium chloride
kb =	kilo bases
mRNA =	messenger ribonucleic acid
μ =	micro
poly A^+ RNA =	polyadenylated ribonucleic acid
poly A^- RNA =	RNA depleted of polyadenylated ribonucleic acid
RIA =	radioimmunoassay
SD =	standard deviation
SEM =	standard error of mean
SBTI =	soybean trypsin inhibitor
OD units =	optical density units

Chapter 1 - Introduction

1.1 General Introduction

The productivity of cattle depends upon the amount of feed they consume and the efficiency with which they convert feed to meat and milk. The importance of feed intake is particularly obvious in dairy cattle production because it is a primary factor limiting milk production (Baile and Della-Fera, 1988). Dairy cows at peak lactation may consume up to four times the amount that they consume prior to parturition. Despite the obvious importance of feed intake in animal production, major gaps exist in our understanding of the mechanisms involved in controlling feed intake. Traditionally feed intake regulation in ruminants is believed to occur primarily at the level of the first stomach, the rumen. Rumen fill, passage rate of digesta through the forestomachs and accumulation of ruminal fermentation products are key components of feed intake control in ruminants (Baile et al., 1988). The gut hormone cholecystokinin (CCK) is one of the major regulators of bile excretion, pancreatic enzyme secretion (Rehfeld, 1989), and is also believed to be an inhibitor of feed intake in monogastrics (Smith, 1984). Due to the traditional belief that intake control in the ruminant was primarily regulated in the rumen research on intestinal feed back mechanisms regulating feed intake has only recently commenced. As a result, data on the role of CCK or other gastrointestinal hormones in ruminants is limited. Glucagon like peptide -1 (GLP-1), a peptide encoded by the proglucagon gene, has been suggested to have a role in central feed intake regulation in rats (Turton et al., 1996). The proglucagon gene is also expressed in the distal small intestine of monogastric species and GLP-1 has been measured in humans (Ørskov et al., 1991), rats (Suzuki et al., 1989) and sheep (Martin and Faulkner, 1996). The expression of the proglucagon gene has not been demonstrated in cattle and its possible role in feed intake regulation has not been investigated. Increased understanding of feed intake regulation in ruminants is a necessary prerequisite to improving production efficiency and optimizing feeding strategies for cattle.

1.1.1 *Molecular Background of CCK*

Cholecystokinin was first described by Ivy and Oldberg (1928) as a hormonal factor stimulating gallbladder contraction. It was first isolated by Mutt and Jorpes (1968) and described as cholecystokinin-pancreozymin for its effect on the gallbladder and pancreatic enzyme secretion. Today, we know that CCK is a peptide hormone and neurotransmitter which is primarily synthesized and secreted by cells in the brain and in the proximal small intestine in response to food ingestion (Rehfeld, 1989). In the rat the transcription unit of CCK is 7 kilobases (kb) and is interrupted by two introns (FIGURE 1.1). Different cell types synthesize CCK peptides of various size but they all apparently derive from a single messenger RNA with between 750 and 850 bases, of which 345 are protein coding (Gubler et al., 1984; Deschenes et al., 1985).

All CCK peptides derive from the 115 amino acid preproCCK protein, consisting of a signal peptide, spacer peptide, the largest CCK form CCK-58, and the COOH-terminal 12-amino acid peptide (FIGURE 1.2). Post-translational modification of preproCCK results in peptides of varying lengths following cleavage of the COOH-terminal 12-amino acid peptide and N-terminal fragments at various sites. In all biologically active CCK molecules the COOH-terminal end includes the tetrapeptide Trp-Met-Asp-Phe-NH_2 and an O-sulphated tyrosyl(SO_3H) (Rehfeld 1989).

The structure of CCK peptides resembles the structure of gastrin peptides. Both share the COOH-terminal pentapeptideamide, and the O-sulphated tyrosine differs only by being in position 6 in gastrin versus position 7 in CCK. Larsson's and Rehfeld's (1977) hypothesis of the peptides' evolution from a common ancestor seems very likely, even though the genes for human gastrin and CCK are located on different chromosomes (Lund et al., 1986).

There are very few differences in the COOH-terminal sequence of preproCCK (amino acid position 86 - 103) across species (FIGURE 1.3) and CCK-8 in bovine plasma has been shown to be identical to human CCK-8 (Dockray et al., 1978).

N-terminal fragments of the CCK-molecule do not have biological activity and

sulphation enhances the biological activity transmitted by the peripheral CCK-A receptor by approximately 100-fold (see also 1.1.3 CCK Receptors and Antagonist). The primary forms of CCK peptides found in blood have been designated CCK-58, CCK-33/39 and CCK-8 (FIGURE 3). Although all of those CCK molecules are found in the peripheral circulation and brain, their abundance appears to vary among species (Dockray et al., 1978; Bloom et al., 1983; Reeve et al., 1986; Eysselein et al., 1990; Eng et al., 1990). These differences in the findings may actually be related to different extraction methods or antibody binding abilities (Reeve et al., 1994). The relative potencies of the various CCK forms remain equivocal (Sakamoto et al., 1985; Reeve et al., 1994).

1.1.2 *Distribution of CCK*

Cholecystokinin peptides are distributed throughout the central nervous system, especially the brain (Dockray, 1976) and periphery, in particular the gastrointestinal tract (Rehfeld, 1986).

In the brain, CCK is localized on synaptic vesicles of nerve terminals, released from synaptosomal preparations by depolarization, possesses binding sites on neuronal elements, depolarizes neurons and is degraded by a selective brain enzyme (Schick et al., 1994). The distribution of CCK-like immunoreactivity in different brain regions is relatively similar for rats, dogs, pigs, cows, guinea pigs, and man (Jansen et al., 1985; Barden et al., 1981; Huang et al., 1986). The highest CCK mRNA abundance and CCK peptide concentrations in the brain are in the cortex, hippocampus, caudate, and thalamus with medium concentration in the hypothalamus. The relative abundance in most brain sites changes in the rat brain throughout development (De Belleroche et al., 1990). The CCK peptides are not capable of crossing the blood brain barrier, and although there are brain regions that lack the blood brain barrier (e.g. area postrema), no penetration of circulating CCK into the hypothalamic extracellular space was observed by Schick et al. (1986). This indicates a separate role of central and peripheral CCK in feed intake regulation.

Distribution of CCK in the gastrointestinal tract is variable across species. In the

intestine of all species, CCK is most abundant in the duodenum and upper jejunum and is synthesized in endocrine cells classified as I cells (Larsson and Rehfeld, 1978). Cholecystokinin is also widely distributed in peripheral neurons (Rehfeld and Lundberg, 1983), with afferent vagal nerve fibers containing CCK (Dockray et al., 1981). Stomach, duodenum, and jejunum contain only a few CCK-containing neurons, whereas the colon contains numerous CCK nerves and colonic smooth muscles have been shown to be excited by CCK (Rehfeld, 1986), thereby altering passage rate of digesta. Rose et al (1996) recently characterized a serine peptidase which specifically inactivates CCK released in the gastrointestinal tract, in particular the CCK of neural origin. They showed that this neuropeptidase is expressed by CCK-responsive neurons and inhibition of its action allows neural CCK-8 to escape inactivation and results in CCK-like effects such as satiation in rodents. Blackshaw and Grundy (1990) showed that in the ferret only the duodenal, not other gastrointestinal tension receptors were excited by CCK-8 intra-arterial injections and also that the vagal mucosal receptors were directly sensitive to CCK-8 which was not affected by cholinergic blockade. These fibers are therefore likely candidates for mediating CCK effects. Pancreatic ganglionic cell bodies and in some species (cat, pig and human) pancreatic islet cells, are also surrounded by CCK nerve terminals (Larsson and Rehfeld, 1979), whereas the pancreatic islets in dog and rat do not have such innervation (Rehfeld et al., 1980). CCK-like immunoreactivity has also been observed in male germ cells (Persson et al., 1989).

1.1.3 *CCK Receptors and Antagonist*

Two different CCK receptors have been identified with peripheral CCK receptors (CCK-A receptor) being distinguishable from central CCK receptors (CCK-B receptor). Selective antagonists have been described and characterized for both receptor subtypes - devazepide (previously MK-329 or L364,718) for CCK-A and L-360,260 for CCK-B (Lotti et al., 1987 and 1989). Cholecystokinin-A receptors have high affinity only for sulfated CCK. They have been located on the pancreatic acinar cells, gallbladder smooth muscle, gastric mucosa, pyloric sphincter, selected areas of the central and peripheral nervous system, and tumoral and neuroblastoma cell lines (Wank et al., 1994). The CCK-

B receptors (which was shown to have identical cDNA sequence as gastrin receptors, Kopin et al., 1994) differentiate poorly between sulphated and non-sulphated CCK and gastrin. They are the predominant brain CCK receptors, but are also present in gallbladder and stomach smooth muscle cells, parietal cells, pancreatic acinar cells of some species, and in some carcinoma cells (Wank et al., 1994). Dourish et al. (1989) found that blocking of the CCK-B receptor could postpone satiety in rats and Schick et al. (1991) reported that the injection of L-365,260 into the lateral hypothalamus increased intake for up to 60 min, thereby providing evidence that meal-induced release of CCK in the lateral hypothalamus occurs during a restricted period of time and may play a role in termination of feeding. Brain sites where CCK suppresses feeding other than the hypothalamus were medial pons and lateral medulla, near where vagal afferent fibers terminate (Schick et al., 1990) and area postrema (Kooy, 1984). Like most gastrointestinal cues the satiety effect of peripherally injected CCK is not only dependent on intact gastric vagal afferent fibers (Smith et al., 1985), but also on the integrity of the nucleus tractus solitarii (NTS) and neural connections from the NTS to the hypothalamus (Crawley, et al., 1984a and 1984b).

1.1.4 *Glucagon Like Peptide-1*

The proglucagon gene has been reported to be expressed in the pancreas, the distal small intestine and to a much lesser extent in selected neurons of the brain (Mojsov et al., 1989; Drucker and Asa, 1988). In the pancreas, proglucagon is predominantly processed to glucagon whereas glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) are major intestinal proglucagon products. In the rat, intestinal perfusion with GLP-2 was shown to increase jejunal glucose uptake (Cheeseman and Tsang, 1996), however, other roles for GLP-2 have not yet been defined. GLP-1 has been studied more intensively and has been shown to be secreted from the L-cells predominantly in the distal ileum (Ørskov et al., 1986). GLP-1 in monogastric species is insulinotropic when blood glucose concentrations are elevated (Holst et al., 1987) and it is also glucagonostatic (Komatsu et al., 1989). Glucagon like peptide -1 has also been shown to inhibit gastric acid secretion (Wettergren et al., 1994) and gastric emptying

(Wettergren et al., 1993). These findings were particularly interesting with respect to non-insulin-dependent diabetes pathogenesis in humans (Willms et al., 1996).

A role for GLP-1 in the central regulation of feeding has also been suggested even though peripherally administered GLP-1 has been reported not to affect feed intake (Turton et al., 1996). Hoyt et al., (1996) demonstrated in rats a decrease of proglucagon mRNA abundance in jejunum (40% of pre-fasting levels) and ileum (20% of pre-fasting levels) during 72 h fasting. During refeeding the jejunal proglucagon mRNA abundance returned to pre-fasting levels, whereas in the ileum no significant increase in proglucagon mRNA abundance occurred. Plasma enteroglucagon and GLP-1 concentrations correlated with the jejunal proglucagon mRNA changes.

Information about the ability of nutrients to induce changes in GLP-1 release, or proglucagon mRNA abundance, are at present limited in monogastrics (Tappenden et al., 1996), although volatile fatty acids and triglycerides appear to increase proglucagon gene expression in intestinally resected rats (Tappenden et al., 1996; Hoyt et al., 1996). The development of radioimmunoassays using antibodies (KMJ-01) specific for the C-terminal end of the biologically active GLP-1 (7-36) amide without binding to the proglucagon fragments released from the pancreas with the secretion of glucagon will help to increase the knowledge with respect to GLP-1 release - however, the antibody binds to the inactive GLP-1 fragment 9-36 (Dr. McIntosh, personal communication) which is produced by the 'di-peptidal peptidase 4' after cleavage of alanine at position 2. Consequently, results obtained by the use of this antibody have to be interpreted with this information in mind.

Very few studies have looked at the function of GLP-1 in ruminants. In sheep, GLP-1 has been shown to induce an increase in blood insulin concentration (Martin and Faulkner, 1993) during hyperglycaemic conditions comparable to that induced by postprandial blood glucose (Martin and Faulkner, 1993). Martin and Faulkner (1996) also demonstrated that somatostatin decreased the circulating GLP-1 concentration in sheep. It has not been established whether proglucagon mRNA abundance in the ileal mucosa or blood GLP-1 concentrations, change throughout fasting or if GLP-1 is released in

response to intestinal nutrients reaching the distal small intestine in nutrients in ruminants.

1.2 Feed Intake Regulation in Monogastrics

Feed intake regulation is an extremely complex process for which information has to be integrated relating to the sensory quality of food, physiological state (pregnancy, lactation, growth, activity, status of nutrient storage), satiety signals from the gastrointestinal system and absorbed nutrients (Read et al., 1994). An appropriate response must then be initiated in order to ensure sufficient intake for maintenance, growth, and production. Most work related to feed intake regulation has been done in monogastric species such as rats (Liddle et al., 1986 and 1988; Garlicki et al., 1990; Reidelberger et al., 1992; Weatherford et al., 1992), dogs (Reidelberger et al., 1989), monkeys (Schick et al., 1987), or pigs (Anika et al., 1981; Ebenezer et al., 1990, Gregory et al., 1989). A large number of eating disorder-related studies have focused on feed intake regulation in humans (Pi-Sunyer et al., 1982; Kissileff et al., 1981; Geracioti and Liddle, 1988; Lieverse et al., 1994). The physiological mechanisms that produce satiety in response to food intake are not clearly defined. It is known that the cells in the gastrointestinal tract respond to changes in nutrient supply and this information is transmitted by neuronal or hormonal pathways to the brain. Central feed intake regulation occurs within certain areas of the brain. A number of gut neurotransmitters are not only signaling information to the brain, but also induce changes within the gastrointestinal tract e.g. CCK influences passage rate of digesta by inducing pyloric sphincter contraction.

1.2.1 *Central Regulation*

The anatomic site of release determines what effect a particular hormone or peptide is going to have on food intake. The forebrain is important in sensory specific satiety, the amygdala and hippocampus play a role in palatability and aversion, and the main areas related to feed intake were identified within the hypothalamus (Silver and

Morley, 1991). The paraventricular nucleus (PVN) and ventro-medial region of the hypothalamus (VMH) act as the satiety control region (stop feeding decision process), and the lateral hypothalamus (LH) region behaves as the feeding control region (start feeding decision process). Stimulation of the LH causes eating, and lesion of the LH causes starvation syndrome (Teitelbaum and Epstein, 1962). In the VMH the reverse results can be seen: stimulation interrupts feeding (Hoebel, 1976), and lesions cause hypothalamic hyperphagia syndrome (Hoebel and Teitelbaum, 1966).

1.2.1.1 Overview

In Hoebel's (1984) review a schematic overview of the involvement of monoamines, opiates, and brain gut peptides in the motivation of feeding is given. The neural systems involved are described as dopaminergic (affecting the LH), adrenergic paths (including noradrenergic acting on the PVN and beta-adrenergic affecting the LH), and serotonergic pathways (modulating the PVN). Briefly, to summarize - after hippocampal and cortical arousal by sensory cues (visual, olfactory, taste) the noradrenergic anti-satiety pathway is activated by the taste of sugar in the mouth and (or) lack of nutrients in the gut and liver, starting at the dorsal vagal complex. This noradrenergic pathway inhibits the PVN-VMH region resulting in reduced satiety. In the situation of previous feed deprival or lack of satiety signals from stomach, intestine, and liver, the LH-beta-adrenergic signal that can inhibit feeding would remain quiescent. In the case of an extended deprivation, a gradual up-regulation of the unstimulated beta-adrenergic receptors could produce an augmented response to satiety factors, which would explain post-starvation anorexia (Jhanwar-Uniyal et al., 1982). As feeding starts, food related responses involving the dopaminergic system are enhanced, and the LH beta-adrenergic signal can then begin to inhibit feeding - satiety can be achieved. The gastric vagal afferent fibers and nucleus tractus solitarii (NTS) are involved in the transmission of the food related responses, e.g. gastrointestinal satiety signals to the hypothalamus (Hoebel, 1984). It has also been demonstrated (Kooy, 1984) that the area postrema is of importance for the CCK mediated satiety.

1.2.1.2 Substances Involved in Feed Intake Control

Several substances have been shown to be involved in feed intake regulation. Cholecystokinin and neurotensin reduce feeding in the rat when injected into the PVN-VMH region implicating the norepinephrine feeding pathway (McCaleb and Myers, 1980; Stanley et al., 1982). However, Voigt et al. (1995) also demonstrated the involvement of a serotonin receptor in CCK-8 induced satiety in rats. Centrally administered insulin enhances sensitivity to CCK (Riedy et al., 1995). Bombesin - also a brain gut peptide - reduces feeding when injected into the LH (Smith et al., 1982), and injection of neuropeptide Y into the PVN region stimulates feeding in the rat (Stanley et al., 1985) via an insulin-dependent mechanism (Schwartz et al., 1992). Different opiates also induce feeding when injected into the PVN (Leibowitz and Hor 1980) intra-cerebroventricularly (McKay et al., 1980), or following peripheral administration (Kumar et al., 1971). Leptin, a substance released from mature adipocytes (Ogawa et al., 1995) was reported to control body weight in mice (Frederich et al., 1995) and leptin receptors were found within the rat brain in the hypothalamus, choroid plexus and cerebellum (Ghilardi et al., 1996; Devos et al., 1996). Leptin has been shown to reduce feed intake in pigs (Ramsay et al., 1997), and rats (Baile et al., 1997) when infused intra-cerebroventricularly. Leptin was recently demonstrated to be expressed in bovine adipose tissue (Ji et al., 1997) and an increase of mRNA abundance in subcutaneous fat was seen during the finishing period of steers.

In addition to the substances mentioned above, other gastrointestinal hormones (somatostatin, motilin, gastrin-releasing peptide, glucagon-like peptide), several pancreatic hormones (insulin, glucagon, pancreatic polypeptide), as well as gonadal steroids have been shown to affect feed intake (Morley, 1987). Estradiol was reported to exert its feed intake-suppressing effect through potentiation of the satiety effect of CCK (Butera et al., 1993).

It cannot be questioned that more than one of these substances aid in the regulation of feed intake control. However, the large evidence for CCK involvement in

satiety regulation, and the highest CCK abundance in the proximal intestine, which is accessible through the duodenal cannulae of available animals, are the main reasons for this research focusing on its role in regulating the feed intake of ruminants. The proglucagon gene, which encodes gastrointestinal peptide glucagon-like peptide (GLP-1) was included in two studies where distal small intestine tissue was accessed through ileal cannulae.

1.2.1.3 CCK

Using the push-pull perfusion technique, Schick et al. (1986) reported that gastric distention in cats induces CCK release in the lateral hypothalamic area and in monkeys. Schick et al. (1987) demonstrated the postprandial release of neuronal CCK-like immunoreactivity in the LH following a carbohydrate/amino acid meal. Cholecystokinin also inhibits feeding when injected into cerebral ventricles with the cerebrospinal fluid serving as an intermediate link between the site of CCK-8 release and its sites of action (Della-Fera et al., 1981). These findings indicate that within the brain, CCK functions as a neural transmitter (Baile and Della-Fera, 1988). Fasting alters brain CCK mRNA abundance and CCK peptide concentration of the right frontal lobe in rats (Greenstein et al., 1990). Sheep fasted for increasing time also required increasing doses of CCK-8 to cause an equivalent decrease in feed intake. These are indications that CCK acts in an adaptive manner to energy deficits (Della-Fera and Baile, 1980b).

1.2.2 *Peripheral Regulation*

The peripheral satiety feeding system is responsible for the termination of meals - that is to create a sensation of fullness so that the animals stop the intake of food (Silver and Morley, 1991). To achieve this, several gut peptides and other stimuli act together. The concentration of CCK in plasma increases during meals, so research focused on the effects of peripheral CCK as a satietin will be further discussed later in this chapter.

1.2.2.2 Circulating CCK Peptides

In order for a CCK peptide to interact with the predominant peripheral CCK-A receptor it requires a sulphated COOH-terminal end of at least the CCK-tetrapeptide (Johnson et al., 1970). Cholecystokinin peptides of differing lengths can be biologically active, whether they consist of 58 or 8 amino acids. The N-terminal fragments of the CCK-molecule have no biological activity (Rehfeld 1986). Due to the structural similarity of the COOH-terminal CCK molecule and the gastrin molecule, technical difficulties associated with cross-reactivity of antibodies raised against CCK and gastrin delayed the development of a radioimmunoassay (RIA) for CCK until the early 1980s. A highly sensitive bioassay using rat pancreatic acinar cells was developed by Liddle et al. (1984). Cross reactivities between CCK and gastrin, and their target cell receptors may have functional significance in some species, depending on the peripheral distribution of the CCK-B receptor which differentiates poorly between sulphated and non-sulphated forms of CCK and gastrin (Wank et al., 1994). In rats the potency of CCK for inducing amylase release from pancreatic acinar cells is about four hundred fold greater than that of gastrin due the fact that only the CCK-A receptor is present so that interference of gastrin in this bioassay is not important (Liddle et al. 1985). Synthetic CCK-8 and CCK-33 isolated from porcine plasma are the main source of CCK used in radioimmunoassay for standard curves. Depending on which antibody is used, all CCK peptides can be detected equally by radioimmunoassay (Beardshall et al., 1992).

Species differences seem to prevail in the major forms of circulating CCK peptide forms. Bloom et al. (1983) showed the elution of two major peaks of CCK-like immunoreactivity from calf plasma, corresponding to CCK-33/39 (40%) and CCK-8 (60%). In canine plasma (Eysselein et al., 1987) CCK-58 is the major circulating form (64%), followed by CCK-33/39 (14%) and CCK-8 (8%). However, the proportion of CCK-58 in blood may be higher than previously reported in other species also, due to CCK-58 being degraded into smaller fragments in plasma and it being less immuno-reactive than other forms of CCK (Reeve et al., 1994). Improvements in the method of extraction of CCK from blood and in antibody design have also contributed to the

detection of longer forms of CCK in the plasma (Reeve et al., 1994). The major site of CCK clearance seems to be the liver, where CCK-8 is eliminated more effectively than the longer forms of CCK (Sakamoto et al., 1985), such that the half life of CCK peptides vary with the length of the molecule. In the plasma aminopeptidases exist that digest the larger molecules of CCK and this degradation can be effectively inhibited by acidification of the blood after collection (Eberlein et al., 1987), but not through the use of the protease inhibitor Aprotinin (Cantor, 1986). Cholecystokinin-8 has a half-life of about 2 min (Cantor et al., 1986), while CCK-33 has a half-life of over 3 min (Gores et al., 1986). Rose et al. (1996) demonstrated recently, that gastrointestinal serine peptidases cleave CCK-8 from the gastrointestinal tract, in particular CCK of neural origin. This finding may explain why the circulating CCK concentrations may not represent the local CCK concentrations within the gastrointestinal tract. The biological significance of the different CCK peptides and their differing half-lives remains under discussion (Reeve et al., 1994).

Mir et al., (1996) reported a correlation coefficient of 0.67 between CCK concentration measured in jugular and portal blood in sheep, which suggests partial hepatic clearance of CCK in ruminants. However, Mir et al. (1996) also found that this correlation coefficient seemed to vary with the source of starch and the resulting duodenal viscosity used in their experiments (Mir et al., 1996, and personal communication). Varying hepatic clearance rates of CCK in response to diet have not previously been reported.

1.2.2.3 Release of CCK

As mentioned above, gastrointestinal CCK is released from the I cells of the proximal small intestine in response to nutrients in the lumen. In several species the release of CCK is known to be induced by different digesta components. In humans, fat, fatty acids, amino acids, protein and carbohydrates are all potent CCK-releasing factors. In rats, amino acids, fatty acids and fat seem to be less effective than protein (Liddle et al., 1986). Iwai et al. (1988) suggested a feed back mechanism in rats involving the level

of active pancreatic protease and a monitor peptide - a peptide produced by pancreatic acinar cells. They proposed a mechanism by which the release of the monitor peptide into the intestinal lumen together with bile and pancreatic enzymes allows the monitor peptide to compete with dietary protein for trypsin and other proteases. During a high protein dietary regime, the monitor peptide is free to enhance CCK release, which in turn leads to higher pancreatic enzyme release. Contrary to Iwai et al.'s (1988) hypothesis, evidence has accumulated that in rats CCK is released in the absence of intestinal trypsin which could not be explained by the interaction of trypsin or the monitor peptide (e.g. after bile diversion from the upper small intestine or luminal washing, Herzig et al., 1994; Miyasaka and Green 1983). Miyasaka et al., (1989) and Lu et al., (1989) demonstrated in rats that an intestinal trypsin-sensitive CCK-releasing peptide was responsible for CCK release. Liddle (1995) proposed that in rats the intestinal trypsin-sensitive CCK-releasing factor spontaneously enhances CCK secretion. Owyang et al. (1986) showed that in humans, trypsin inhibits the rise in plasma CCK that is stimulated by either intra-duodenal amino acids or fat, indicating that the level of active duodenal protease in man is also involved in a feedback mechanism for CCK release. Liddle (1995) supported this theory suggesting that in humans an intestinal trypsin-sensitive CCK-releasing factor requires stimulation by intra-luminal nutrients such as amino acids and fatty acids, instead of the spontaneous action of the intestinal CCK-releasing factor in rats. There is evidence that a similar feedback mechanism exists for several other species, including chickens, rats, guinea pigs, dogs, and humans (Chernick et al., 1948; Green and Lyman, 1972; Kim et al., 1995; Shiratori et al., 1989; Owyang et al., 1986). However, monitor peptide- or CCK releasing factor analogues have not been isolated in humans, or any other species.

Herzig et al. (1994) demonstrated an involvement of somatostatin in modifying CCK release in bile-pancreatic juice-diverted rats, which supports the hypothesis that paracrine influences of other inhibitory regulators influence the regulation of pancreatic secretions.

1.2.2.4 Functions of CCK

Since the early characterization as a substance that caused gallbladder contraction, a number of functions of CCK within the gastrointestinal system have been identified. Cholecystokinin regulates pancreatic exocrine secretion potently and Rehfeld (1989) showed that the amount of CCK released during meals seems sufficient to account for almost all enzyme secretion. The secretion of most pancreatic enzymes is stimulated by CCK. Pancreatic amylase, chymotrypsinogen, trypsinogen are only some of the enzymes that are regulated by CCK (Niederau et al., 1994; Soudah et al. 1992). Intra-venous infusion of CCK and intra-duodenal infusion of soybean trypsin inhibitor, increase the expression of chymotrypsinogen and trypsinogen genes in the rat pancreas, whereas the expression of the genes encoding pancreatic amylase and ribonuclease is decreased (Rosewicz et al., 1989). In rats soybean trypsin inhibitor has also been shown to stimulate CCK release and increase the abundance of CCK mRNA, which follows an increase in transcription of the CCK gene (Liddle et al., 1988). This process is in part regulated through the above mentioned monitor peptide and trypsin-sensitive CCK-releasing factor and likely reflects transcriptional regulation of intestinal polypeptide hormones. When soybean trypsin inhibitor is included in the diet of humans the activity of duodenal protease is altered, but these changes are not correlated to the plasma CCK level (Holm et al., 1988a and b).

As mentioned previously, pancreatic endocrine secretion is controlled by CCK even though there are considerable species differences (Rehfeld et al., 1980). In humans, pigs, and young calves the pancreatic islets are innervated with neural terminals that release small molecular forms of CCK (CCK-4), but in dogs and rats the pancreatic islets do not have such innervation. In species which do have the CCK-neurons (cats, humans, pigs, and calves) the blood CCK-8 and CCK-33 are not effective in releasing insulin and glucagon, but CCK-4 is an effective releaser of insulin and glucagon. In species without the CCK-nerve terminals (rat, and dog) CCK-33, CCK-8, and cerulein stimulate the release of insulin and glucagon, whereas CCK-4 is essentially without

effect on insulin and glucagon secretion (Rehfeld et al., 1980; Otsuki et al. 1979; Hermansen, 1984). This may be explained by the relative importance of pancreatic enzymes with respect to diet composition across species; for example, the rat and dog consume extremely protein rich diets, whereas humans and pigs consume more carbohydrates and therefore may have developed a separate mechanism for the control of insulin and glucagon release. The role of CCK in digestion and the mechanism controlling its release seem to be very complex and differ across species which is to be expected as animals have evolved with markedly different dietary preferences and requirements.

Cholecystokinin peptides are known to have a trophic effect on pancreatic growth, which may be important during early development of the pancreas (Guilloteau et al., 1992; Le Meuth et al., 1993), and appear to stimulate intestinal blood flow (Thulin and Sannegård, 1978). Inhibition of gastric emptying by relaxation of the proximal part of the stomach and stimulation of the pyloric sphincter (Yamagashi and Debas, 1978) is another function of CCK in most monogastrics, even though there seem to be species differences with the cat's pyloric sphincter being relaxed by CCK (Behar et al., 1979). Recently, CCK has been implicated with decreasing intestinal glucose uptake by down-regulating jejunal transepithelial hexose transporters in rats (Hirsch et al., 1996) a process apparently mediated by CCK-A receptors.

In 1973 Gibbs et al., reported that peripherally administered CCK inhibits food intake in rats. This effect has subsequently been demonstrated in several other species including pigs (Anika et al., 1981; Ebenezer et al., 1990, Gregory et al., 1989), monkeys (Gibbs and Smith, 1977), and humans (Kissileff et al., 1981, Lieverse et al., 1994). Different mechanisms have been proposed to explain how peripheral CCK could exert its satiety actions (Reidelberger, 1994), which will be discussed in the next section in more detail.

1.2.2.5 Peripheral CCK Satiety

Mechanisms that produce satiety in response to food intake are not clearly

defined. Cholecystokinin regulates pancreatic exocrine secretion and CCK may also influence hexose transport (Hirsch et al., 1996). As a result CCK can potentially influence the rate of digestion within the intestine as well as the rate of absorption of nutrients from the intestine. By increasing motility in the large intestine (Rehfeld, 1986; Blackshaw and Grundy, 1996), CCK can also affect digesta passage rate. However, CCK is believed to also have some more direct satiety actions.

Gibbs at al. (1973) first reported that CCK administration suppresses feeding in rats and since then much has been learned about the mechanisms involved. Highly specific CCK-receptor antagonists allowed detailed studies on the role of endogenous CCK and several reviews summarize our present understanding of CCK control of feeding behavior (Smith, 1984; Baile et al., 1986; Morley, 1987; Silver and Morley, 1991; Pirke et al., 1993; Ritter et al., 1994; Reidelberger, 1994; Read et al., 1994; Smith and Gibbs, 1994). Most of the studies focus on CCK as a satiety factor alone. However, it is apparent that several other gastrointestinal hormones interact in synergistic or antagonistic fashion to regulate feed intake control (Hinton et al., 1986; Younes et al., 1989, Silver and Morley, 1991). The details of the interactions of CCK with other gastrointestinal hormones in the control of satiety is beyond the scope of this literature review. The reader is referred to the reviews of Baile et al. (1986) and Lee et al. (1994).

Evidence for a changed CCK response in obese vs. lean volunteers has been presented in several papers (Lieverse et al. 1994; Kissileff et al., 1981; Pi-Sunyer et al. 1982). Cholecystokinin may also be involved in some eating disorders such as anorexia or bulimia nervosa (Philipp et al. 1991) although this remains equivocal (Pirke et al. 1994). This literature review will describe the mechanisms of CCK induced satiety cues, within the 'normal' population of animals.

Two mechanisms have been suggested for the inhibitory effect of cholecystokinin on food intake: a central action of brain CCK on the brain feeding system (Schick et al., 1987), and a peripheral action of gut cholecystokinin mediated by abdominal vagal afferent nerves. Peripheral CCK cannot pass the blood-brain barrier, which is why it is believed to produce peripheral satiety mechanisms. Indirect evidence demonstrates that

CCK released from the upper small intestine in response to luminal nutrients requires involvement of abdominal vagal nerves because total vagotomy blocks CCK induced inhibition of food intake in rats (Smith et al., 1985). As mentioned above, CCK release in the hypothalamus occurs during food ingestion. As such it is possible that centrally released CCK induces changes within the gastrointestinal system, similar to peripherally released CCK. Della-Fera et al. (1990a) investigated the effect of intra-peritoneal (i.p.) and intra-lateral-ventricular (l.v.) CCK-8 injections on gastric emptying rate and feed intake of liquid and solid diets in rats. Gastric emptying was significantly reduced after i.p. injections of CCK-8 (4 μg/kg) in sham feeding (digesta does not enter the stomach, but is removed by esophageal cannula preventing gastric distention) as well as in real feeding. Feed intake was also reduced after CCK injections, but 18 hours after administration the difference compared to the control group was no longer significant. The l.v. injections of CCK (0.05 or 0.5 μg/kg) did not affect gastric emptying rate in gastric cannulated or intact rats. This finding indicates that suppression of feeding after central administration of CCK is not caused by a centrally mediated effect on gastric emptying. Feed intake was reduced only for real feeding after l.v. injections of CCK and sham feeding remained unaffected confirming that peripheral CCK-mediated satiety mechanism do not seem to be produced by centrally released CCK.

Several hypotheses try to explain the mediation of the peripheral CCK satiety mechanism. Ingested food can act locally by a paracrine mechanism, at a distance by an endocrine mechanism. Gut neuronal CCK may also be released by food and act locally to initiate satiety. All of these theories will be discussed in detail.

Paracrine Mechanism

Using the definition of peripheral satiety as inducing a feeling of fullness, the stomach is the prime sensory candidate. One of the commonly accepted effects of CCK is the ability to delay the rate of gastric emptying due to the contraction of the pyloric sphincter which contains CCK receptors in the smooth muscle layer. The delayed rate of gastric emptying leads to an increase in gastric fill which is monitored by stretch sensors.

The information is mediated through the vagal afferent fibers to the brain, resulting in a decrease in feed intake (Moran and McHugh, 1982). This mechanism is commonly referred to as the gastric satiety action of CCK. Green et al. (1988) used the CCK receptor antagonist devazepide to study the effect of endogenous and exogenous CCK on gastric emptying in the conscious rat. They found that the delay of gastric emptying which was induced by a peptone rich diet, protease inhibitor-containing diet, or direct intra-venous CCK infusion, could be reversed by the use of the CCK antagonist, while the hydrochloric acid and hyperosmolar saliva effect on gastric emptying remained unchanged. Moran et al. (1988) also investigated gastric and nongastric mechanisms for satiety action of CCK in rats. They found that sham feeding was inhibited by intra-peritoneal CCK injection and vagotomy blocked only part of the satiety effect of CCK. Moran et al. (1988) concluded that 28% of the satiety effect of CCK cannot be explained by CCK-inhibition of gastric emptying rate that is mediated through the afferent vagal fibers. Therefore other pathways have to be involved. One possibility is that circulating CCK-levels mediate information to the area postrema (where no blood brain barrier exists) where peripheral CCK-A receptors have been located (Kooy 1984) and could transfer the information regarding circulating CCK-levels to the satiety center.

Endocrine Mechanism

If CCK produces satiety by an endocrine mechanism, it is important that the criteria for physiological satiety effect of a gut hormone established by Smith (1984) are being met. These are:

1 - the hormone has to be released during a meal

2 - the exogenous administration of the hormone has to induce satiety

3 - the endogenous release of the hormone has to induce satiety

4 - antagonists or other treatments have to attenuate the satiety induced by exogenous or endogenous hormone

5 - concentrations of the hormone occurring during or after a meal have to be sufficient or necessary for normal satiety to occur.

Most of the above criteria have been met for CCK even though it is still under debate whether the CCK concentrations released after a meal are sufficient and necessary for satiety to occur.

In rats (Reidelberger et al., 1989; Della-Fera et al., 1990a; Yox et al., 1992) and pigs (Ebenezer et al., 1990) it has been shown that exogenous and endogenous CCK influence feed intake. Through the use of CCK receptor antagonist injection prior to CCK administration, the suppression of feeding induced by CCK was attenuated. This has been interpreted as a satiety effect due to CCK, even though the pathway used to mediate the satiety effect was not specified. Studies that reported satiety effects of CCK sometimes used pharmacological doses and may have induced malaise and aversion instead of true satiety (Swerdlow et al., 1983). Reidelberger and Solomon (1986) showed that the doses of CCK required to inhibit feeding in rats are 3 to 5 times larger than maximal doses for stimulation of pancreatic enzyme secretion of CCK, suggesting that postprandial plasma levels of CCK are possibly too low to produce satiety by themselves. Linden et al. (1989) observed that postprandial plasma levels of CCK in rats were similar to those produced by an inhibitory intra-peritoneal dose of CCK that reduced feeding, suggesting that CCK is a physiological hormonal mediator. However, Reidelberger (1994a) suggested that intra-peritoneal injections of CCK may cause paracrine effects before being absorbed into circulation, thereby questioning intra-peritoneal injections as a means to validate a physiological role of CCK.

In human volunteers, Lieverse et al. (1994a, b, and c) reported that intra-venously injected CCK induced satiety without nausea. The CCK concentrations within the plasma did remain within the normal postprandial range and the effects of CCK could be reversed by the CCK-A receptor antagonist loxiglumide. However, the satiety effects induced by CCK were very weak and it is questionable whether the postprandial circulating CCK release is sufficient and necessary for satiety to occur.

Kaplan et al. (1994) tested the involvement of gastric emptying and post-gastric signals in the satiation of rats by removal of gastric contents through gastric cannulae.

They found that the rats increased their feed intake to accurately replace the amounts of food withdrawn from the stomach such that net intake at the end of three subsequent withdrawals did not differ from that ingested during the initial test. They concluded that the animals integrated signals from post-gastric as well as gastric sources.

Supporting the hypothesis of endogenous CCK inducing post-gastric satiety signals, the majority of results regarding satiety effect of endogenously released CCK during sham feeding trials indicate the involvement of CCK-A receptors. However, in orally ingested feeding the results are equivocal. In pigs, emulsified fat, monoglycerides, glycerol, glucose, and oleic acid infused into the duodenum reduced feed intake, but the effect could only be attenuated by CCK-A receptor blockage for fat and monoglyceride (Gregory et al., 1989). The effect of duodenally infused corn oil on feed intake in humans could not be reversed (Drewe et al., 1992) nor could the effect of oleic acid be attenuated in rats (Woltman and Reidelberger, 1993). Differences in duration of feed deprivation prior to feeding, the amount of CCK antagonist administered, or species could explain the discrepancy in the findings. Due to differences in the experimental design among experiments other feed intake-reducing factors could have been triggered that could not be reversed with CCK antagonist, e.g. gastric fill may complicate the picture compared to sham feeding. Moreover, the CCK antagonist also permeates the blood-brain barrier, and could therefore act on brain CCK receptors.

Reidelberger et al. (1994) used a monoclonal antibody and combination of CCK-A and CCK-B receptor antagonists to evaluate the mode of transmission used for CCK signals regarding food intake or pancreatic secretion in the rat. Their conclusion was that CCK stimulates pancreatic enzymes at least partially by an endocrine mechanism and produces satiety by non-endocrine mechanisms. They concluded this because immuno-neutralization of circulating CCK had no effect on food intake under the same conditions and in the same animals in which CCK-A receptor blockade stimulated feeding. These findings are in agreement with the study of Greenberg and Smith (1988) which demonstrated a major reduction in CCK-8 satiety in hepatic compared to intra-peritoneal infusion, which supports the paracrine or neurocrine path of CCK satiety action.

Neurocrine Mechanism

Rehfeld (1989) reviewed the distribution of CCK containing neurons and concluded that the stomach and proximal small intestine contain very few CCK nerves although the origin of the intestinal CCK fibers has not been determined. With a low abundance of CCK-containing neurons in the small intestine, the stimulation of the vagal afferent fibers through CCK released from neuron endings seems to be less likely than through paracrine mechanisms. Moreover, as mentioned above Rose et al. (1996) characterized a serine peptidase that specifically inactivates CCK-8 of neural origin.

Even though the complete mechanisms involved in the transmission of peripheral CCK satiety signals are not clear, it can be concluded that paracrine or neural mechanisms are the major pathways that stimulate the vagal mechanisms. However, vagal mediation does not appear to be the only way endogenous CCK signals satiety cues and further research will be needed to specify the mechanisms involved. These mechanisms seem to differ among species

1.3 Feed Intake Regulation in Ruminants

Ruminants are able to utilize a forage-based diet by relying on rumen microbes to aid in the fermentation of fiber particles that mammals lack the enzymes to digest. Their first two stomachs the rumen and reticulum (reticulorumen) serve as a 'storage container and incubator' to maintain favorable conditions for the microbial fermentation of the feed. About one half of the diet is digested in the rumen and some fermentation products, e.g. volatile fatty acids and ammonium ions can be absorbed through the rumen papillae (Chase et al., 1977). After a certain particle size is reached digesta passes to the second and third stomach (reticulum and omasum) and from there to the abomasum, the equivalent of the monogastric stomach.

Regulation of feed intake in ruminants has been believed to be a function of rumen capacity, and rumen fill with feed characteristics determining the amount of feed that can be accommodated in the rumen as well as the mean retention time in the

reticulorumen (Conrad, 1966). Recently, evidence is accumulating that the animal exerts control over the outflow of digesta from the reticulorumen and that control of digesta outflow occurs after the reticulum omasal orifice (Malbert and Ruckebusch, 1989; Mathison et al. 1995).

Modern feeding strategies in cattle promote the inclusion of feedstuff that are rumen-inert and are available for digestion and absorption in the small intestine. Often referred to as bypass feedstuff, they allow for supplementation of energy or protein to the animal without affecting the rumen microbes and without the microbes utilizing and altering the feedstuff. The most commonly used bypass products are bypass-protein and bypass-fat, but sources of carbohydrates which are degradable in the rumen to a varying extent are also important. Inclusion of this rumen inert feed is not only a means to synchronize energy and protein supply, but it also improves efficiency by shifting the site of digestion from the rumen to the intestine. With an increasing amount of nutrients being absorbed from the small intestine relative to those absorbed from the rumen, it has to be expected that the signals from these parts of the gastrointestinal tract contribute to the satiety mechanisms in the ruminants. In order to differentiate between ruminal and intestinal satiety signals, abomasal or duodenal infusions appear to be the method of choice. Research has shown, for example, that gastrointestinal infusion of fatty acids suppresses dry matter intake in cows (Christensen et al., 1994).

Knowledge about nutrient absorption from the intestine and how these intestinal satiety signals are involved in regulating feed intake in ruminants are limited at present and an improved understanding of these mechanisms would allow for improved feeding strategies thereby being of benefit to the commercial cattle industry.

1.3.1 *Central Regulation*

It has long been known that ruminant animals can dramatically increase their voluntary feed intake in peak lactation - in some cases up to four times that observed in the non-lactating animal (Baile and Della-Fera, 1988). Prolonged (more than six weeks) administration of somatotropin has been shown to lead not only to an increased milk

production, but also to increased feed intake (Chilliard, 1989). The magnitude of the increase in feed intake is believed to depend upon the increase in milk yield, degree of body condition change and nutrient density of the diet (Burton et al., 1994). However, circulating somatotropin concentrations are not believed to affect food intake directly (Forbes, 1980).

The stimulated feed intake cannot be attributed solely to a change in feed characteristics, but is likely also caused by gastrointestinal and metabolic factors. The increased metabolic demand is possibly resulting in increased feed intake in response to central integration of information of the status of body stores.

The central feed intake regulation in ruminants appears to be similar to that of monogastrics with the PVN and LH regions of the hypothalamus integrating information regarding satiety. Some differences between species exist, however, because beta adrenoreceptor agonists reduced feeding in rats, but stimulated feeding in both sheep and cattle (Baile, 1975). Similar to monogastric central feed intake regulation, neuropeptide Y and CCK are neurotransmitters that are involved in the brain satiety regulation as Della-Fera et al. (1990) demonstrated in sheep that administration of neuropeptide Y into the third ventricle induced feeding whereas cerebroventricular administration of CCK-8 decreased feed intake. Sheep fasted for increasing time also required increasing doses of CCK-8 to cause an equivalent decrease in feed intake, which indicates that it acts in an adaptive manner with energy deficits of the animal (Della-Fera and Baile, 1980b). Intra-cerebroventricular injection of sheep with CCK antibodies was also reported to stimulate feeding and daily food intake (Della-Fera et al., 1981). Opioid peptides and CCK have both been shown to alter rumen motility after central administration (Bueno et al., 1983; Della-Fera and Baile, 1980a) and there is evidence that central CCK influences duodenal motility in sheep (Cottrell and Iggo, 1984). Changes in methionine enkephalin have been observed in specific areas of the hypothalamus of fed and fasted sheep (Scallet et al., 1985). Central dynorphin injections were demonstrated to override satiety signals produced by an inflated balloon in the rumen or propionic acid infused into the rumen (Della-Fera et al., 1990b). Intra-cerebroventricular injection of CCK-8 also caused a

decrease of plasma insulin concentration, which would impact feed intake (Della-Fera and Baile, 1985). These findings strongly support the involvement of neuropeptide Y, opioid peptides and CCK in the integration of signals regarding the control of food intake in ruminants.

1.3.2 *Forestomach Regulation*

For wild herbivores, their fibrous diets are often relatively difficult to harvest, so that bite size and rate of biting are important factors determining feed intake. The development of the forestomach system allows microbes to aid in fermentation of the fibrous material, but the long period of storage makes the physical capacity of the reticulorumen a potential factor limiting intake. For decades the concept of feed intake control in ruminants has been that the animal's attempts to maximize rumen fill and that feed characteristics determine retention time of feed in the reticulorumen (Forbes 1995). Optimizing microbial fermentation efficiency through synchronization of the availability of fermentable carbohydrates and protein to the microbes by selecting appropriate feed sources would be one way of managing the feed characteristics. Several factors which are believed to control feed intake in the forestomach system are discussed next.

1.3.2.1 Distention

In domesticated ruminants the relationship between intake and digestibility, rate of digestion, fiber content and retention time of digesta in the rumen makes the rumen capacity limiting for feed intake on low quality forage diets (Conrad et al., 1964). By increasing the digestibility of forages, Ørskov et al. (1991) found that intake can be increased due to the positive relationship between digestibility of food and intake. Stretch receptors within the reticulorumen wall monitor the level of distention (Grovum, 1979) and send satiety signals via the vagus nerve to the brain when a certain physical capacity has been reached in a manner similar to monogastric stomach distention satiety signals. These specific meal-limiting physical factors may be of importance when food is available only for a limited time during the day or when feed characteristics do not allow for the passage of digesta through the digestive tract. If the digestibility is above 67%,

however, the animal with prolonged access to feed is able to compensate for the dilution of digestible energy to meet its energy requirements, unless the physiological demands on the animal are very high, for example in peak lactation (Baile and Della-Fera, 1988). Experiments that demonstrated the effect of the distention of the rumen and reticulum included placing bulky objects like water filled balloons into the rumen and recording the following stimulation of the medulla oblongata within the brain (Leek, 1986). Welch (1967) introduced indigestible polypropylene fibers into the rumen which depressed voluntary intake. The reduced intake was only temporal as intakes returned to normal levels after about 30 days providing the fibers were small enough to be ruminated, which was therefore less likely to obstruct the passage of digesta. Distention of rumen or reticulum is therefore a limiting factor of feed intake under some conditions. This is consistent with the concept that characteristics of feed determine the amount of material which can be accommodated within the forestomach and the mean retention time.

Total extent of digestion in the whole alimentary tract is not sufficient to give an adequate understanding of the physical limit to intake. The total tract digestibility may be an indication of the large intestine's capacity of digestion. However, the extent to which food particles are digested in the rumen is a more important determinant of feed intake because Carro et al. (1991) demonstrated a significant correlation ($r = -.84$) between the intake and neutral detergent fiber (NDF) content of forages. With highly digestible feed having a small particle size there is evidence that the intake is controlled to meet energy requirements. This means that feed intake regulating mechanisms other than rumen capacity must dominate (Baumgardt, 1970).

1.3.2.2 Temperature

Temperature receptors within the abdomen wall of sheep have been implicated in depressed intake in ruminants (Forbes, 1994). Heating coils in the rumen (Gengler et al., 1970) and addition of warm water to the rumen of cattle decreased intake, whereas addition of cold water to the rumen of cattle caused increased intake of a pelleted diet (Bhattacharya and Warner, 1968). Considering these findings, the fact that temperature receptors have not been identified in the wall of the reticulorumen, and that Kennedy et

al. (1986) reporting increased feed intake in response to cold temperature exposure of sheep, it appears more likely that changes in blood temperature that result from altered rumen content temperature are transmitted to the medulla oblongata, where the temperature centres are located. The changes in feed intake will likely result from the central integration of temperature related information with other satiety cues.

In warm climates this is an important factor influencing feed intake, but even in moderate climates the addition of feedstuff with relatively low heat increment like fat can be preferred over others such as carbohydrates and protein in order to maximize digestible energy intake (Palmquist and Conrad, 1983). Kennedy et al. (1986) showed that exposure to cold temperature enables the animal to increase its voluntary intake, which is an indication for the animal exerting feed intake control instead of feed intake being determined by feed characteristics.

1.3.2.3 Osmolarity

The rumen appears to be sensitive to the osmolarity of the digesta within the lumen, especially when this is elevated by additions of salts and other osmotically active substances, which was reviewed by Grovum (1995). This is an important feed intake limiting factor during dehydration of animals (Utley et al., 1970). Due to the fact that the blood tonicity changes in response to altered rumen osmolarity it is possible that these signals also contribute to satiety. However, Ternouth and Beattie (1971) demonstrated that abomasal and ruminal sodium chloride loads both effect jugular tonicity, but only ruminal loads depressed intake. These findings makes the wall of the reticulorumen a likely mediator for the anorectic properties of ruminal sodium chloride loads, even though the putative osmoreceptor mediating the intake effect still needs to be identified.

1.3.2.4 Volatile Fatty Acids

Volatile fatty acids (VFA) are major fermentation products of ruminal microbial digestion of feed. They are absorbed from the rumen and have been suggested to be involved in feed intake control. Baile and Forbes (1974) showed that both acetate and propionate decrease feed intake in cows, sheep and goats. The receptors mediating the

suppressive effect of acetate on feed intake are located at the luminal side of the rumen (Baile and Mayer, 1969), whereas those for propionate are located in the liver (Anil and Forbes, 1980). De Jong (1986) questioned the involvement of volatile fatty acids due to the fact that under physiological conditions a spontaneous meal is neither preceded or followed by changes in blood VFA. He criticized several experiments using infusion of VFAs into jugular, hepatic portal, ruminal vein, or ruminal lumen for exceeding the rate of physiological VFA production (inducing malaise instead of satiety), short term monitoring of effects of infusion, simultaneous increase of sodium concentrations (altering osmolarity and VFA concentration at the same time), or extreme changes occurring locally prior to mixture within the lumen. Grovum (1995) also criticized experimental designs involving infusions of VFAs into the lumen or intra-venously because osmotic pressure was not controlled where saline served as a control. The direct feed intake-reducing effect of propionate has also been questioned because propionate is a potent stimulator of insulin (Peters et al., 1983) and the effect on feed intake could be attributed to insulin. During once or twice a day feeding, marked increases in VFA concentrations can be observed in the blood, which may contribute to feed intake regulation.

Farningham and Whyte (1993) separated the satiety effects due to hypertonia from mechanisms regulated by VFAs or their metabolites using equimolar saline as controls for portal infusion of propionate and acetate in sheep. Due to the fact that propionate depressed food intake more so than equimolar doses of acetate, mannitol or isotonic saline, and the inconsistent effect of propionate on insulin, they concluded that portal propionate flow has a potential role in control of food intake in ruminants which is independent of osmotic effects or changes in plasma insulin concentration.

Farningham et al. (1992) reported the synergistic effect of CCK and propionate in controlling feed intake, using portal infusion rates where propionate or CCK by themselves failed to affect feed intake but the concommittant infusion of both significantly reduced the intake of *ad libitum* fed sheep. These data indicate that there may be situations when isolated components of satiety signals are not sufficient to reduce

feed intake, but that there is a need to look at the effect of the combinations of satiety cues.

1.3.2.5 Forestomach Motility and Orifice Activity

Mathison et al. (1995) reviewed the contribution of the propulsive activities of the forestomach to the flow of digesta and thereby the control of feed intake. In agreement with Ulyatt et al. (1986) they concluded that the major rate-limiting step in clearing dietary residue from the reticulorumen is passage through the reticulo-omasal orifice, which appears to be controlled by the animal's response to feed and environment rather than by feed characteristics. The omasal fill appears to limit the emptying from the reticulum, abomasal fill impacts on the outflow from the omasum, and duodenal fill seems to regulate the digesta flow out of the abomasum. Malbert and Ruckebusch (1989) investigated the effect of pylorectomy on food intake in hay fed adult sheep and found that the removal of the pyloric sphincter increased abomasal outflow by more than 200% and food intake by 48%. If the retention time of digesta and feed intake was determined by feed characteristics this should not be the case. It can be concluded that a feed intake response occurs as a result of a combination of feed characteristics, gastrointestinal, and metabolic satiety signals.

1.3.2.6 Other Factors

The possibility of fermentation products absorbed from the reticulorumen locally inducing the release of intestinal satiety signals like CCK has not been evaluated in the ruminant. Considering the importance of ruminal fermentation products and that dietary preferences in monogastric species possibly alter the release mechanisms for CCK, this hypothesis would justify investigating the CCK mRNA abundance in the forestomach system.

Ji et al. (1997) showed that the leptin gene is expressed in adipose tissue of cattle and they found that the mRNA abundance increased during the finishing period of steers. Seoane et al. (1972) demonstrated the existence of humoral factors causing satiety in sheep using a cross circulation experiment. Insulin and glucagon are both released during

meals and in response to propionate or butyrate infusions (Basset 1972) and both have been implicated as having an intake reducing effect (Deetz and Wangsness, 1981).

Since it is to be expected that intestinally-absorbed nutrients also contribute to the release of humoral factors, these satiety effects will be discussed together with the intestinal regulation

1.3.3 *Intestinal Regulation*

Due to the only recently commencing interest in satiety signals derived from the intestinal system of ruminants, information on the role of CCK in gastrointestinal function in ruminants is limited.

1.3.3.1 Release and Functions of CCK

The endocrine changes in the developing ruminant have been studied for some time. Guilloteau et al. (1992) found increased plasma CCK, gastrin, secretin, and VIP within the first 28 h of neonate calves in response to colostrum feeding, possibly stimulating the development of the pancreas and gastrointestinal system. Bloom et al. (1983) reported CCK and pancreatic polypeptide, insulin, and glucagon release in response to intra-venous infusion of bombesin or gastrin releasing peptide in pre-ruminant calves. Gastrointestinal regulatory peptides are therefore important in the young ruminant and likely do not lose their functions in the digestion of an adult ruminant after the development of the forestomach. However, more research is needed to clarify their possible interaction with gastrointestinal satiety signals.

A systematic experimental approach to investigate the extent to which nutrition influences CCK release has not been applied yet in ruminants, however, there is evidence that amino acids and lipids are involved. The increasing use of rumen inert feed, mostly protein and lipids, can be expected to influence plasma CCK concentration by selectively delivering nutrients to the intestine. Thus the effects of these diets on plasma CCK and feed intake, as well as pancreatic enzyme secretion is of interest.

Furuse et al. (1991) monitored postprandial plasma CCK concentration in dairy

cows and observed relatively stable CCK values over a 6 hour test period. They suggested that a relatively continuous flow of digesta from the rumen to the duodenum was responsible for the stable CCK values within the 6 hour postprandial range of observation. However, evidence that significant duodenal changes can occur in both flow and amino acid composition of digesta reaching the intestine (Gill et al. unpublished data; Robinson and Kennelly, 1991) throughout a day implies the need for monitoring the CCK blood concentrations over a longer period of time.

In goats, Furuse et al. (1992) showed that duodenal infusion of certain amino acids, in particular phenylalanine and tryptophan, increases the release of CCK, whereas other amino acids did not induce any change in plasma CCK concentration. Choi and Palmquist (1996) demonstrated that high fat diets increase plasma CCK and pancreatic polypeptide, decrease plasma insulin, and suppress feed intake in lactating dairy cows. Christensen et al. (1994) found that abomasal infusion of long-chain fatty acids also suppressed dry matter intake in cows.

Evidence was presented by Kato et al. (1986) that intra-duodenal soybean trypsin inhibitor increases pancreatic secretion in sheep which the authors suggested to be transmitted by CCK, though CCK plasma concentrations were not measured. These findings would support the hypothesis that pancreatic proteases are involved in a feedback mechanism to control the release of CCK in ruminants. Bainter et al. (1993) contradicted previous assumptions that the anti-nutritional proteins of soybean flour were inactivated by forestomach fermentation by showing that they passed intact through the intestine. As a consequence CCK may mediate the information about the need of additional pancreatic proteases with soybean flour diets.

Pancreatic exocrine secretion in ruminants appears to be stimulated by CCK to a similar extent as in monogastrics, as demonstrated by intra-duodenally infused soybean trypsin inhibitor and intra-venously infused CCK affecting pancreatic enzyme secretion in sheep and pre-ruminating calves. Intra-venously administered CCK-8 and intra-duodenally infused soybean trypsin inhibitor increased pancreatic juice volume, total protein output, and trypsin concentrations in pancreatic juice (Kato et al., 1986; Harada et

al., 1986)). Kato et al. (1986) found that atropine reduced the pancreatic secretion and eliminated the stimulating effect of intra-duodenally infused soybean trypsin on pancreatic enzyme secretion whereas the intra-venous CCK-8 infusion effect was not abolished by atropine administration. It was concluded that the release of CCK is dependent on the cholinergic pathway, but its effect on the exocrine pancreas is not. Contradictory to these conclusions, it has been shown in monogastrics that only doses of CCK producing supra-physiological concentrations of blood CCK are independent from the cholinergic pathway, but that the CCK concentrations that are comparable to postprandial plasma levels are abolished by atropine blockade (Soudah, et al. 1992). Kato et al. (1986) did not measure plasma CCK concentrations in their experiment; however their CCK infusion may have induced a supraphysiological CCK plasma concentration and as a consequence the effects produced by CCK infusion could not be abolished by atropine blockade. Infusion of soybean trypsin inhibitor would be expected to only produce a physiological CCK concentration which would be dependent on the cholinergic pathway. Harada et al. (1986) found that intra-venous infusion of CCK increased both pancreatic juice flow and protein concentration, but did not alter hepatic bile flow. Zabielski et al. (1992) showed that pancreatic juice volume, protein output and trypsin content were all increased in response to intra-duodenal infusion of soybean extract infusion. However, both atropine and cold vagal blockade eliminated the stimulation. No significant increase in circulating CCK could be found after duodenal soybean extract infusion, but pancreatic juice was not re-introduced into the duodenum and therefore the feedback mechanism could not function.

Zabielski et al. (1994) also investigated the influence of site of CCK-8 infusion (jugular vein and gastro-epiploic artery) and found that local administration of CCK-8 caused a much faster and stronger pancreatic response than peripheral administration. Vagal blockade decreased pancreatic response for local, but not for peripheral administered CCK-8. It was concluded that CCK-8 stimulates exocrine pancreas by two different mechanisms: a direct and vagus-independent and an indirect and vagus-dependent effect in the duodenum. Zabielski et al. (1995) also demonstrated that intra-

duodenal infusion of CCK-8 stimulates the pancreatic enzyme release.

Mineo et al. (1994) reported that a continuous intra-venous infusion of CCK-8 (3 to 1000 pmol kg^{-1} min^{-1} for 30 min) induced a dose-dependent increase in plasma insulin, but did not alter plasma glucagon. The increase of insulin was seen two and five minutes after the start of CCK infusion and the threshold of CCK was 10 to 30 pmol kg^{-1} min^{-1}, but was no longer significant after 10 min. Plasma glucose concentrations were decreased 10 min after start of the CCK infusion and remained reduced until 10 min after the end of the infusion, which could be an effect of the increased insulin secretion.

These findings confirm the role of CCK in regulating pancreatic exocrine secretion in ruminants, which will indirectly impact feed intake regulation through alteration of intestinal digestion and availability for absorption. The effect of CCK on the expression of pancreatic enzyme genes or other intestinal genes has so far only been studied in monogastrics.

Husar et al. (1996) reported that increased intestinal viscosity of different diets decreased CCK secretion in sheep, which may be an indication of feed characteristics impacting on the intestinal endocrine response.

1.3.3.2 Peripheral CCK Satiety

As defined above, a peripheral short term feed intake-regulating factor is involved in the termination of a meal and induction of a fullness sensation. Being released in response to a meal is one of the criteria that according to Smith (1984) would have to be met in order for a physiological satiety action to occur. In ruminants, in spite of the forestomach system buffering the immediate postprandial responses, the same principles would apply.

As discussed above Furuse et al., (1991) could not detect any changes in plasma CCK concentration following a concentrate or forage meal of dairy cows.

McCarthy et al. (1992) monitored changes of gastric inhibitory polypeptide (GIP) and other metabolites in response to feeding following a 48 h fasting period in sheep. An

increase in GIP and several metabolites at different times after feeding was observed, but the study did not look at changing CCK plasma concentration. The plasma triacylglyceride concentration increased after three hours, which could have increased the plasma CCK concentration at this stage.

Few studies have investigated the direct peripheral CCK satiety effect in ruminants. Grovum (1981) demonstrated that intra-venous infusions of CCK affected the motility of the reticulo-rumen and feed intake in sheep, which supports the finding of Symons (1978) of suppressed feed intake in nematode-infected hungry sheep after intra-venous CCK-8. Neither of these studies looked at induced circulating CCK blood concentrations with the infusions, which makes it difficult to say if the doses they infused were within a physiological range. Honde and Bueno (1984) found that centrally administered CCK decreased feed intake in sheep but could not confirm the anorectic effect of intra-venously given CCK-8 and neither injection altered rumination.

Farningham and Whyte (1993) observed that after infusing 18 pmol kg^{-1} min^{-1} CCK-8 into the portal vein no effect on feed intake was observed, even though reticular contractions were reduced by 35%. The infusion of propionate at 1.2 mmol min^{-1} did not affect feed intake or reticular motility. In combination these two treatments reduced the 2h feed intake dramatically, even though the 24 h feed intake was not altered. Reticular motility was reduced by 41%. It was concluded that CCK and propionate act synergistically to induce a satiety response in sheep. The concentration of CCK infused according to Mineo et al. (1994) induces an insulin response, and propionate releases insulin at 1.2 mmol min^{-1}, also (Farningham and Whyte, 1993). According to Grovum (1995) a moderate increase of insulin reduces intake in sheep. The reduction in feed intake could therefore be due to the secondary insulin response, which was not measured during this experiment.

Choi and Palmquist (1996) found that in lactating cows, high fat diets increased CCK and pancreatic polypeptide concentrations and decreased plasma insulin concentration and feed intake. Choi et al. (1996) also reported that the feed intake-suppressing effect of endogenous CCK released in lactating cows on a high fat diet could

be reversed by the CCK-A receptor antagonist MK-329 for two hour feed intake. This report of a CCK effect being reversed by a CCK-A receptor antagonist supports the hypothesis of CCK involvement in feed intake control in cattle.

1.4 Conclusion

Intestinal satiety mechanisms are very likely involved in feed intake regulation in ruminants similar to their function in monogastric species. The forestomach system provides satiety cues and buffers extreme postprandial changes in the intestinal digesta composition, but the use of rumen-inert feeds that selectively deliver nutrients to the intestine enhances the importance of intestinal satiety cues. Increased understanding of feed intake regulation in ruminants will facilitate the development of feeding strategies to increase production efficiency, or provide possible selection criteria to improve feed intake related traits in cattle.

Objectives of this Research

The objective of this research was to provide new information about the physiological mechanisms controlling feed intake in ruminants. An increased understanding of feed intake regulation will facilitate the development of more effective dietary management strategies and allow cattle to fully realize their genetic potential for meat and milk production. Several experiments were carried out with the following goals:

1. to determine the effect of feed restriction and different physiological states (lactating vs. non-lactating) on CCK gene expression and CCK release in ruminants, as well as evaluate the horizontal distribution of CCK mRNA abundance in the ruminant digestive tract.
2. to develop an in vivo method to study bovine intestinal response to dietary manipulation at the molecular level.
3. to determine the effect of feed restriction and refeeding on the expression of the CCK and proglucagon gene and CCK and GLP-1 release.
4. to determine the effect of nutritional stimuli supplied to the rumen on the expression of the CCK and proglucagon genes, CCK and GLP-1 release, and feed intake.

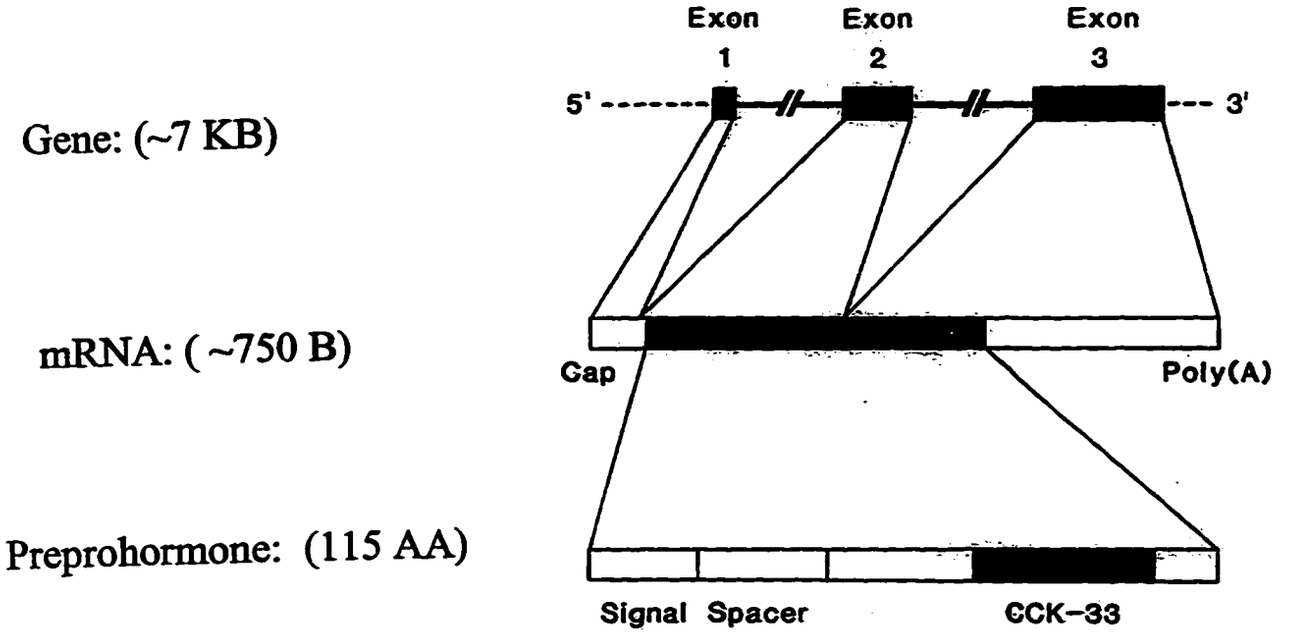

FIGURE 1.1 - Schematic Illustration of Overall Structure of the Rat CCK gene, CCK RNA, and its Primary Translation Product PreproCCK.

Boxes 1-3 on the gene are the exons separated by introns, of which the first has a size of 1 kilobase (kb) and the second a size of 5 kb. The mRNA has a size of 750 bases, of which 345 are protein coding. Location of the sequence for the first isolated CCK peptide (CCK-33) in preproCCK is shown (from Rehfeld, 1989, with permission).

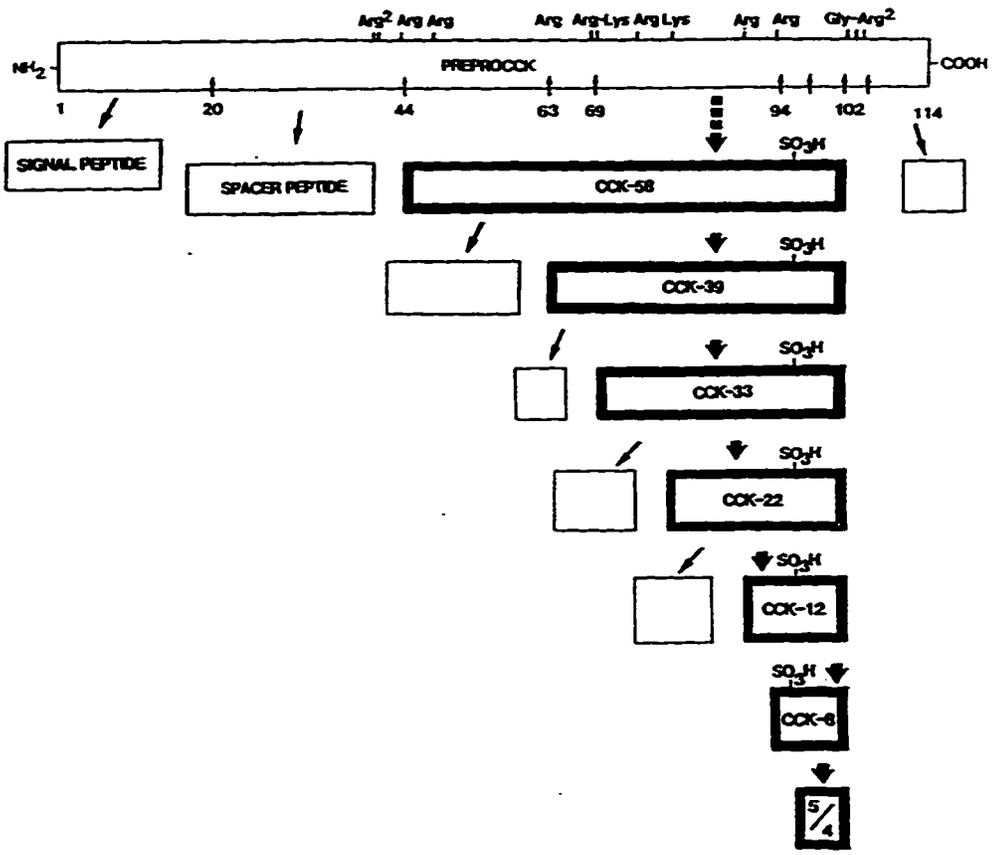

FIGURE 1.2 - Presumed Post-Translational Processing of PreproCCK in Intestinal I Cells.

Mono- and dibasic cleavage sites are indicated on porcine preproCCK molecule. Fragments framed in bold lines are biological active forms containing COOH-terminal carboxyamidated tetrapeptide, Trp-Met-Asp-Phe-NH_2, and O-sulphated tyrosyl$_{96}$(SO_3H). Broken arrow, occurrence of several intermediate forms during processing (From Rehfeld 1989, with permission).

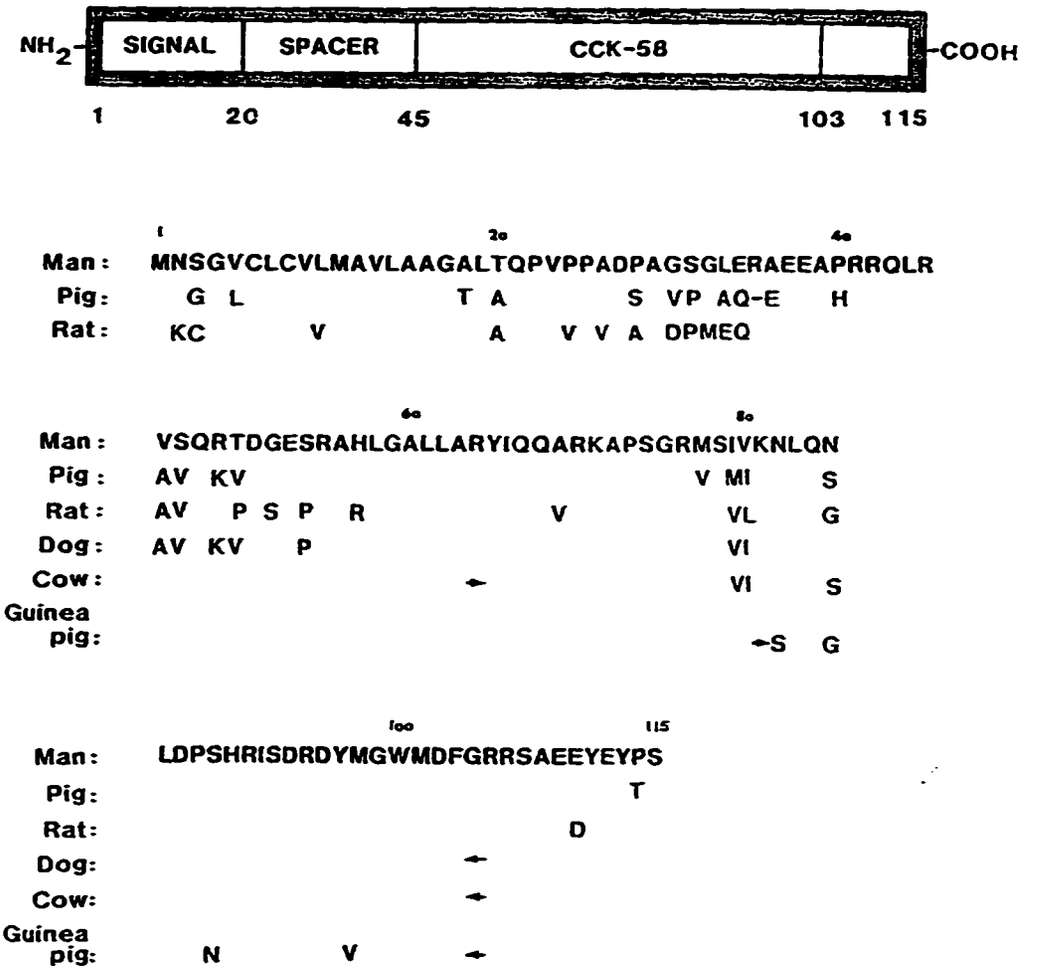

FIGURE 1.3 - Primary Structure of PreproCCK and Known PreproCCK Fragments from Different Mammals.

The first NH_2-terminal 20 amino acids constitute the signal peptide. The following spacer sequence of about 25 amino acids has a considerable number of substitutions between the species examined so far. It is from this region that all known biological active forms of CCK originate. Within the sequence of the CCK molecule CCK-8, only the guinea pig shows differences from the human sequence. Vertical arrows, onset and termination of sequenced dog, cow and guinea pig peptides. Only the guinea pig shows sequence differences in the region of the COOH-terminal site of the CCK molecule (Rehfeld, 1989, with permission).

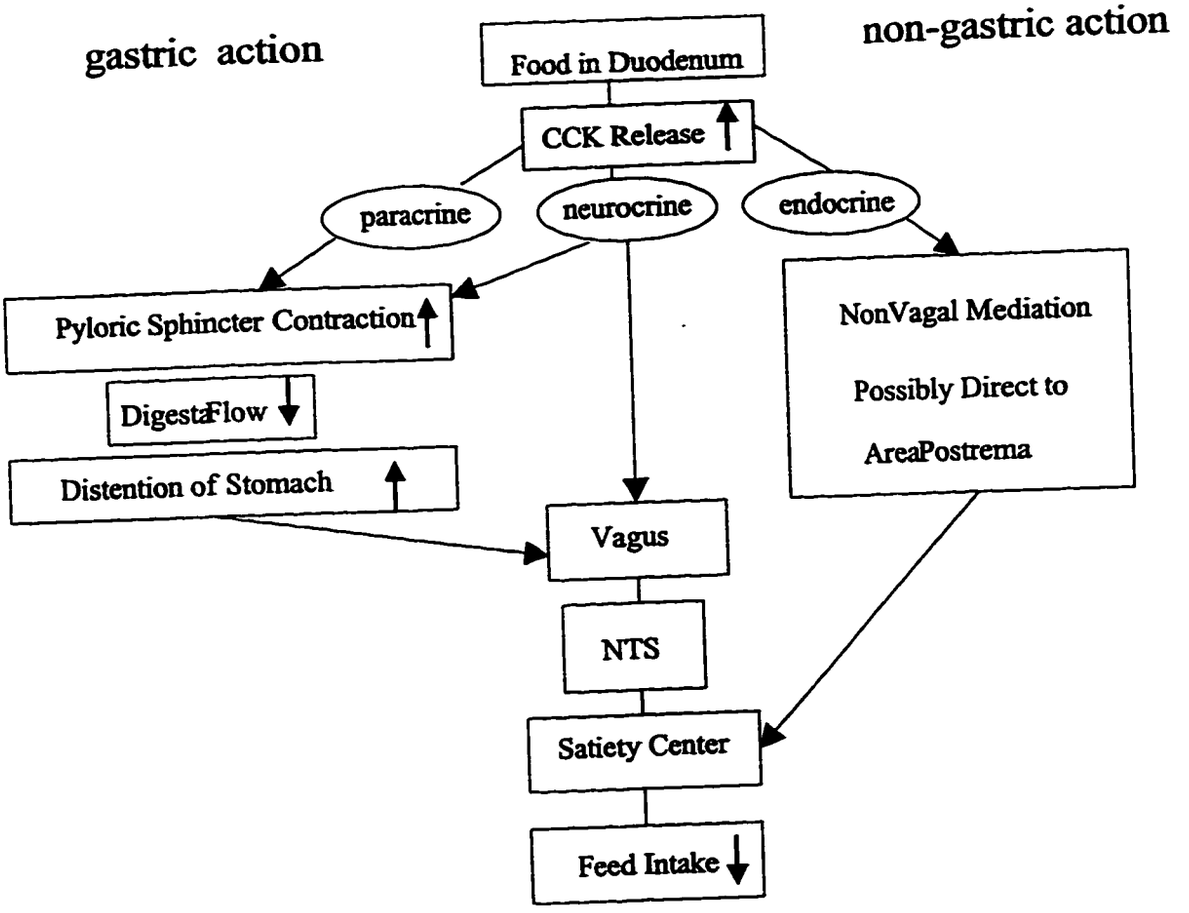

FIGURE 1. 4 - Gastric and Non-gastric Actions of CCK

CCK is released in response to nutrients reaching the intestine. The released CCK can act by paracrine, neurocrine or endocrine mechanisms. The gastric action of pyloric sphincter contraction, reduced gastric emptying, and subsequent increased gastric fill is mediated by paracrine or local neurocrine action. Non-gastric action of CCK is either mediated endocrine by circulating CCK peptides reaching the area postrema, where CCK-A receptors are located, which can mediate the information about circulating CCK concentrations to the satiety center, or by neurocrine mediation through the afferent vagal fibers, activation of the nucleus tractus solitarii (NTS) to the satiety center.

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Chapter 2 - CCK Protein Measurement Using Radioimmunoassay - Comparison of Two Extraction Methods

2.1 Introduction

The ability of CCK to induce gallbladder muscle contraction, or release amylase from pancreatic acinar cells was used to identify it as a gastrointestinal hormone and later to measure cholecystokinin concentration in blood or tissue (Mutt, 1980). Liddle et al. (1984) developed a specific and sensitive bioassay for measuring plasma CCK, based on amylase secretion from isolated rat acini, which was used until the early 1990s (Rosewicz et al., 1989; Sharara et al., 1993). Although Liddle et al. (1984) showed that pancreatic acinar cells receive their stimulation almost exclusively from CCK, the question of other gastrointestinal hormones interfering with a bioassays, possibly through other signal pathways, is warranted.

Antisera raised specifically against one hormone are used in radioimmunoassays (RIA) to measure the concentration of the hormone with little or no cross-reactivity with other hormones. The development of a sensitive RIA for cholecystokinin has historically been relatively difficult as a result of the following two complications. The isotope labeling of CCK was demonstrated to be challenging due to oxidation conditions during traditional labeling methods which do not allow preservation of biological activity, and structural similarities between the C-terminal end of the CCK peptides and peptides of the gastrin family that made it difficult to raise antisera specific to CCK (Rehfeld, 1978). Sankaran et al., (1979) demonstrated that conjugation of CCK to the Bolton Hunter reagent allows the maintenance of the immunoreactivity, which was tested by the ability of the labeled CCK to bind to pancreatic acinar cells. Although other methods of labeling CCK peptides were reported (Rehfeld, 1978; Praissman et al., 1982) the finding of Sankaran et al. (1979) led to the commercially available synthetic, sulfated CCK octapeptide (CCK-8s) labeled with ^{125}I by the Bolton Hunter method.

Different antisera were raised against CCK peptides for use in RIA, some of which were raised against the N-terminal end of the peptides and had high cross reactivities with degraded or unsulphated - therefore not biologically active - CCK peptides, whereas other antisera raised against the C-terminal end of CCK, were also recognized by gastrin peptides (Rehfeld 1979; Walsh et al., 1982; Schafmeyer et al., 1982; Bloom et al., 1983; Chang and Chey, 1983; Turkelson et al. 1986; Eysselein et al., 1987). Cantor and Rehfeld (1985) and Cantor (1986), reviewed the performance of several antisera in RIA and heterogeneity of CCK peptides in different species was studied by Jansen and Lamers (1985) taking advantage of differences in antisera characteristics.

More recently antisera were raised against the C-terminal end of CCK with negligible cross-reactivity against gastrin peptides (Byrnes et al., 1981; Hashimura 1982; Jansen and Lamers, 1983; Beardshall et al., 1992) and have since been successfully used in CCK RIA. Common features of most of them are that they use a 72 h incubation period with antiserum, tracer and sample and dextran coated charcoal to separate free and bound antibody, similar to the conditions used in the CCK RIA for this thesis.

Cholecystokinin peptides have been extracted from tissues in boiling water but the peptides are also soluble in organic solvents like methanol (Eng et al., 1982). Separating the hydrophobic plasma fraction from other, possibly interfering, plasma proteins can be done by several methods, two of which are chromatography or organic solvent extraction. Jansen and Lamers (1983) reported that hormone-free plasma (after charcoal removal of all active peptides) induced non-specific interference in the CCK radioimmunoassay, which could be abolished by extraction with ethanol. Extraction with ethanol and subsequent centrifugation precipitates the plasma protein fraction not soluble in ethanol and allows the hydrophobic supernatant to be decanted.

Eysselein et al. (1987) reported that C^{18} Sep-Pak cartridges could successfully be used to prepare plasma for a CCK radioimmunoassay. The C^{18} Sep-Pak cartridges chromatography allows the hydrophobic peptides to bind to a non-polar C^{18} Sephadex matrix, to wash out plasma protein fractions that do not bind to the matrix, and elute the

desired proteins with an increasingly non-polar gradient like acetonitrile.

Both methods of extracting CCK fractions from plasma were compared to determine the method to be used to analyze the plasma samples collected for the experiments reported in this thesis.

2.2 RIA Protocol

The CCK antiserum (Deno), donated by Dr. J. Calam, was raised by immunizing a rabbit against CCK 33. It appears to be identical to the antiserum A2, validated previously (Beardshall et al., 1992) in Dr. Calam's laboratory, in every respect of cross-reactivity with CCK peptides and gastrin peptides. This means that it binds all sulphated CCK peptides larger than CCK-8 to a similar extent, but the cross-reactivity to gastrin or non-sulphated CCK peptides is reduced by approximately 100 fold, so that non-biologically active peptides do not interfere with the assay. Cholecystokinin antiserum (Deno) was used at a final dilution of 1:1,000,000 in .05 M sodium phosphate buffer (pH 7.4) containing .01 M EDTA, .15% gelatin (wt/vol), and .02% sodium azide (wt/vol). Synthetic, sulfated CCK octapeptide labeled with ^{125}I by the Bolton Hunter method (Amersham International, Buckinghamshire, Canada), was used as tracer with 1000 cpm in each assay tube. The total volume per assay tube was 1 ml. After incubation at 4°C for three days, free and bound tracer were separated by the addition of 100 μl sodium phosphate buffer containing 6% (wt/vol) charcoal (Norit-A grade, Fisher, Edmonton, Canada) and .3% (wt/vol) dextran (Sigma Chemical Company, St. Louis, MO, USA). Incubation time from addition of charcoal to separation by centrifugation (10 min, 4°C, 1500 g) was 5 to 6 min, limiting the number of tubes per assay to less than 50 samples run in duplicates plus standard curve. Subsamples (800 μl) of the supernatant were counted in a gamma counter. Inhibition curves were set up using the following synthetic peptides; sulphated CCK octapeptide (CCK 8s, Sigma Chemical Company), non-sulfated CCK octapeptide (CCK 8ns, Sigma Chemical Company), and porcine CCK tritriacontapeptide (CCK 33s, Peninsula Laboratories, CA, USA).

2.3 Plasma Extraction with C^{18} Sep-Pak Cartridges

Eysselein et al. (1987) reported CCK-58 to be the main circulating form of cholecystokinin in dogs using C^{18} Sep-Pak Cartridges after acidification of collected blood to prevent *in vitro* degradation of large CCK peptides. To evaluate the extraction efficiency of the method we used C^{18} Sep-PAK cartridges (Fisher, Edmonton) which had a wider funnel above the C^{18} Sephadex column which hold 15 instead of 3 ml, in order to facilitate the application of plasma samples during the experiments.

2.3.1 Blood Sampling

Blood was collected from indwelling jugular catheters. Plastic syringes were used to collect the blood, which was immediately transferred into pre-cooled glass tubes containing .25 ml ice cold sodium acetate buffer (pH 3.6) ml^{-1} blood. The sodium acetate buffer was prepared according to Eysselein et al. (1987) by adding, 30 ml glacial acetic acid, and 63 ml 1M NaOH into a total volume of 1 l of .17 N NaCl. Plasma was separated within 30 minutes by centrifugation and kept on ice until C^{18} Sep-Pak extraction. Five milliliters of plasma were used, which included 1 ml of sodium acetate buffer, thus for further calculations 5 ml of mixture was considered equivalent to 4 ml of plasma.

2.3.2 C^{18} Sep-Pak Extraction

Plasma from the acidified blood (5 ml) was slowly mixed with three volumes (15 ml) of 2% trifluoroacetic acid (TFA) (vol:vol) and stored at 4°C until further extraction.

The C^{18} Sep-PAK cartridges were arranged on a solid phase extraction vacuum manifold (Cat. # 5-7250, Supelco, Bellefonte, P.A., USA) and were activated with 10 ml acetonitrile, washed with 10 ml water and equilibrated with 10 ml .1% TFA (vol:vol). Twenty milliliters of the plasma/TFA mixture was applied to the column at a flow rate of approximately 2 ml min^{-1}. Cartridges were washed with 10 ml of .1% TFA (vol:vol) and the hydrophobic fraction, which contained the CCK peptides was eluted into 5 ml glass assay tubes with 2.5 ml of 50:50 acetonitrile and .05% TFA (vol:vol). The eluate was

mixed and one half transferred into another assay tube.

The eluates were dried in a rotary evaporator overnight at room temperature and stored until at -20°C until reconstitution in assay buffer immediately before the RIA. The time the samples remained at room temperature was minimized to avoid degradation of CCK peptides.

2.3.3 Parallelism

In order to assess the ability of the Deno antiserum to bind CCK peptides isolated from acidified bovine plasma using C^{18} Sep-PAK cartridges several parallelism tests were performed on the eluate of different samples. Two eluates were pooled to increase the CCK concentrations, to allow the first measured sample to be about 40 to 50% specific binding. The samples were resuspended in 1600 μl assay buffer and were serially diluted so that 800 to 50 μl sample were included into an assay as described above. The specific binding was measured, plotted against the CCK concentration and compared to the inhibition curves of sulphated and non-sulphated synthetic CCK-8 peptides and porcine CCK-33.

2.3.4 Recovery of ^{125}I-labeled CCK-8

Blood was collected and acidified, plasma separated by centrifugation, and 5 ml plasma diluted with 2% TFA (vol:vol) as described above. Different concentrations (160,000 cpm = 10 pM, n=5; 40,000 cpm = 2.5 pM, n=5; 10,000 cpm = 0.6 pM, n=8; and 4000 cpm = 0.25 pM, n = 5) of ^{125}I-labeled CCK-8 were added to the plasma/TFA solution and mixed. An aliquot of .5 ml was removed from each sample before extraction to be counted for 10 min in a gamma counter. The mixture of labeled CCK, plasma and TFA was processed through the pre-activated C^{18} Sep-PAK cartridges. The 2.5 ml eluate was split into .5 ml aliquots and counted in a gamma counter to avoid volume-related differences in counting efficiency of the gamma counter (FIGURE 2.1). Aliquots (.5 ml) of the plasma TFA mixture loaded onto the column, the .1% TFA washes, the tubes the plasma/TFA/label were mixed in before loading the column, and the C^{18} Sephadex fill of

the cartridges were counted in a gamma counter in order to trace the fate of the labeled CCK. The extraction efficiency was calculated from total counts added to the C^{18} Sep-PAK cartridges (counts of .5 ml aliquot*39) divided by the total counts extracted (total of the eluate aliquot counts) and expressed as a percentage.

2.3.5 Quality Control and Intra- and Inter-assay variation

Internal standard samples were obtained by extracting 100 ml of bovine blood using the C^{18} Sep-Pak extraction method. The eluates were pooled, aliquots evaporated, and stored at -20°C until resuspension for the assay. A total of 25 radioimmunoassays with 50 to 100 tubes per assay were completed with the blood samples collected during the experiments of this thesis. Duplicate internal standard samples and duplicate experimental samples were included in all assays. Assays were completed using the same batch of iodinated CCK and synthetic CCK-8s standard aliquots from the same weighing.

The following parameters were monitored for all assays: nonspecific binding, maximal binding, estimated dosage for 20, 50, and 80 % binding inhibition, and specific binding of the internal standard sample. An intra-assay variation coefficient was calculated from the percent error between all duplicate samples within the assays. The inter-assay variation coefficient was calculated from the standard deviation of the internal standard samples between assays divided by the mean dose value.

2.4 Plasma Extraction with Ethanol

Jansen and Lamers (1982) found that plasma extraction with methanol, acetone, or ethanol could reduce the non-specific interference within their CCK radioimmunoassay. Neither methanol nor acetone extraction had improved recovery over the more cost- efficient ethanol extraction, and as such ethanol was chosen. Cantor (1986) showed, that the protease inhibitor Aprotinin was not beneficial for reducing the *in vitro* degradation of CCK peptides, even though the aminopeptidase inhibitor bestatin did reduce degradation occurring at room temperature. Cantor (1986) also demonstrated that when kept in an ice bath, the degradation of either CCK-33 or CCK-8 was negligible

for up to three hours. Thus, none of the protease inhibitors were added to the blood collected for the presented experiments. Cantor (1986) also demonstrated that EDTA, although reducing the antigen binding at higher concentrations was the anticoagulant of choice if the plasma had to be frozen, because heparin caused precipitation after thawing.

2.4.1 Blood sampling

Blood was collected from indwelling jugular catheters. Plastic syringes were used to take the blood, which was immediately transferred into pre-cooled glass tubes containing 5 μmol ethyleno-diaminitetraacetate (EDTA) ml^{-1} blood and kept on ice until centrifugation. Plasma was separated within 30 minutes by centrifugation, frozen, and kept at -80°C until ethanol extraction.

2.4.2 Ethanol extraction

To extract the ethanol-soluble fraction of plasma proteins, containing CCK, the frozen plasma was thawed and two aliquots of 1.5 ml were added to 3 ml of 98% ethanol (vol/vol) in 12 ml glass test tubes. The mixture was vortexed for 10 sec, centrifuged at 2000 g for 15 min, and the supernatant decanted into a 5 ml glass assay tube. After rotary evaporation overnight the dried extract was stored at -20°C until reconstituted in assay buffer for the RIA. As mentioned above, the time the samples spent at room temperature was minimized to avoid degradation of CCK peptides.

2.4.3 Parallelism

In order to assess the ability of the Deno antiserum to bind CCK peptides isolated from bovine plasma by ethanol extraction parallelism tests were performed using the eluates of different samples. After resuspended in 1600 μl assay buffer the samples underwent serial dilution so that 800 to 50 μl sample were included into an assay as described above. The specific binding was measured, blotted against the CCK concentration, and compared to the inhibition curves of sulphated and non-sulphated synthetic CCK-8 peptides and porcine CCK-33

2.4.4 Recovery of ^{125}I-labeled CCK-8

Blood was collected into EDTA and plasma extracted as described above. Aliquots of 3.0 ml plasma aliquots were placed into 15 ml glass test tubes. Different concentrations (160,000 cpm = 10 pM, n=4; 40,000 cpm = 2.5 pM, n=4; 10,000 cpm = 0.6 pM, n=6; and 4000 cpm = 0.25 pM, n = 4) of ^{125} I-labeled CCK-8 were added, and mixed. An aliquot of .5 ml was removed from each sample before extraction to be counted for 10 min in a gamma counter. Five milliliters of 98% ethanol (vol/vol) was added to each plasma sample and the extraction completed by vortexing and decanting. The supernatant was divided into 5 aliquots of .5 ml to avoid volume-related counting inefficiencies in the gamma counter (FIGURE 2.1). Efficiency of extraction was calculated using total counts added (counts of .5 ml aliquot *5) divided by total counts extracted (total of all eluate aliquot counts) and expressed as a percentage.

2.4.5 Quality Control and Intra- and Inter-assay variation

In order to have internal standard samples, 100 ml of bovine blood was extracted by the ethanol extraction method. The supernatants were pooled, aliquots evaporated, and stored at -20°C until resuspension for the assay. A total of 18 RIAs with 80 to 100 tubes per assay were completed with the blood samples collected during the experiments describing this thesis. Duplicate internal standard samples and duplicate experimental samples were included in all assays. Assays were completed using the same batch of iodinated CCK and synthetic CCK-8s standard aliquots from the same weighing.

To monitor the accuracy of each RIA nonspecific binding, maximal binding, estimated dosage for 20, 50, and 80% binding inhibition, and specific binding of the internal standard sample were assessed. An intra-assay variation coefficient was calculated from the percent error among all duplicate samples within the assays. The inter-assay variation coefficient was calculated from the standard deviation of the internal standard samples between assays divided by the mean dose value.

2.5 Results and Discussion

The CCK-8s peptide was weighed in and used for both extraction methods as standard to calculate the concentration of hormone in the analyzed blood samples. The hormone concentrations are expressed as CCK-8 equivalent pmol l^{-1}. Equal binding of all human CCK peptides to the antibody (Deno) has previously been demonstrated (Beardshall et al., 1992) and is likely also present for bovine CCK peptides (Bloom et al., 1983). A binding test was performed by adding 1000 cpm of iodinated CCK tracer and Deno antiserum (titer 1:1,000,000) to synthetic CCK-8 peptides. This showed the specific bound ratio to be 0.6, which is in agreement with the results from Dr. Calam's laboratory. Nonspecific binding was between 6 and 10% of maximal binding. The detection limit of the assay, defined as the lowest concentration of CCK in the assay tube which could be differentiated from the absence of CCK in the tube with 96% confidence was .2 pmol l^{-1}.

The inhibition curves produced with the CCK-8ns, CCK-8s, and CCK-33 are shown in FIGURE 2.2. In contrast to the results obtained in Dr. Calam's laboratory which indicated that CCK33 and CCK-8s had nearly congruent inhibition curves (Beardshall et al., 1992) we observed a shift in CCK-33 inhibition curve (less sensitive) although it remained parallel to the CCK-8 inhibition curve. The half maximum inhibition of binding of tracer to antibody was 2.7 pmol l^{-1} for CCK 8s and 29.8 pmol l^{-1} for CCK-33. The half maximum inhibition of binding of tracer to antibody by CCK-8 was 1000 pmol , which is in agreement with the information from Dr. Calam's laboratory. Non-specific binding, percentage specific bound, estimated dosage 20, 50, and 80 remained constant throughout the samples from either method. The internal standard sample measured approximately 53.5% specific bound for either method of extraction.

2.5.1 C^{18} Sep-Pak Extraction

The evaporated eluates from different cows, or when taken from the same animal at different times showed discolouration varying from dark brown to light yellow, which could not be explained. To assess if the discolouration affected the binding of the CCK

proteins to the antibody, differently discoloured samples were included to test parallelism.

The parallelism test showed that Deno antiserum binds to CCK peptides extracted from bovine plasma by Sep-Pak extraction in a similar manner as it binds to the synthetic CCK peptides. Bovine CCK peptides have an identical amino acid sequence to human CCK forms within the C-terminal region of biologically active peptides (Rehfeld et al., 1986) which is where the Deno antiserum binds. From figure 2.3. it can be seen that the discolouration of the eluates did not affect the binding of the antibody to CCK, as parallelism was maintained for all samples.

The extraction efficiency of iodinated CCK-8 with the Sep-Pak method was 80.62% (±2.78%) [mean (SEM)]. FIGURE 2.4 summarizes the extraction efficiency data. It was apparent, that the individual cartridges' extraction efficiency differed more than expected, compared to the extraction efficiencies reported from Dr. Calam's lab with 87% (±0.93%) for C^{18} Sep-Pak cartridges from a different supplier (Waters Association, Milford, MA, USA), which did not have the wider funnel cartridges that allowed application of 15 instead of 3 ml.. As mentioned above the eluate of each extracted plasma sample was split into halves to obtain 'duplicates', instead of separating the duplicates with separate columns, due to limited space on the vacuum manifold and the cost of the Sep-Pak cartridges. Unfortunately this meant that the individual cartridge extraction efficiency variation, as observed during the labeled CCK-8s recovery tests, increased the variation between samples. The farm laboratory facilities were not licensed for use of radioactive markers and therefore no CCK-8s labeled with tritium could be used as an internal marker in order to calculate the individual column recovery for each sample.

Monitoring the fate of the iodinated tracer, it was demonstrated that approximately 10 - 15% of the total counts added to the column with the plasma did not bind to the C^{18} Sephadex matrix when applied to the column. In part this was due to 5 - 10% free ^{125}I not attached to CCK (Amersham technical service information by phone). The amount of label which was washed from the column with .1% TFA (vol/vol),

attached to the column after eluation, or remained attached to the tube the plasma, TFA and labeled CCK were mixed in, was below 1% each. This is an indication that the low extraction efficiency is not due to label binding to glass or plastic during the extraction process.

The intra-, and inter-assay coefficient for the assays run with samples extracted with Sep-Pak cartridges were 8.2% and 8.0% respectively. Although the intra-assay coefficient includes the variation due consistency of extraction efficiency, this is not the case when the already extracted eluate is divided in halves.

2.5.2 Ethanol Extraction

No major discolouration differences could be seen in the evaporated supernatant after the ethanol extraction and evaporation. The parallelism test showed (FIGURE 2.5) that Deno antiserum binds to CCK peptides extracted by from bovine plasma by ethanol extraction in a similar manner as it binds to the synthetic CCK peptides.

The extraction efficiency of iodinated CCK-8 with was 91.38% (.44) [mean (SEM)]. FIGURE 2.4 summarizes the extraction efficiency data. Our resulting extraction efficiencies were higher than the data reported from Dr. Calam's laboratory using the ethanol precipitation method [84% (±0.4%)], although the standard error of means was comparable between both laboratories, which reveals a consistent extraction efficiency.

The intra-, and inter-assay coefficient for the assays run with samples extracted with ethanol precipitation were 7.7% and 8.2% respectively. Due to the separate extraction of each duplicate the intra-assay coefficient included the variation due to extraction efficiency inconsistencies.

2.6 Conclusion

The larger variation of extraction efficiency for the C^{18} Sep-Pak extraction method compared to the ethanol extraction method was the main reason for choosing the latter method to analyze the blood samples collected in the experiments conducted for this

thesis. Moreover the ethanol extraction method proved to be logistically easier to use during experiments which involved a large number of blood samples collected within a short period of time. This was due to the fact that ethanol extracted plasma samples could be frozen and stored until extraction, whereas the plasma samples for the C^{18} Sep-Pak cartridge extraction needed to be extracted immediately, which was challenging with limited space on the vacuum manifold.

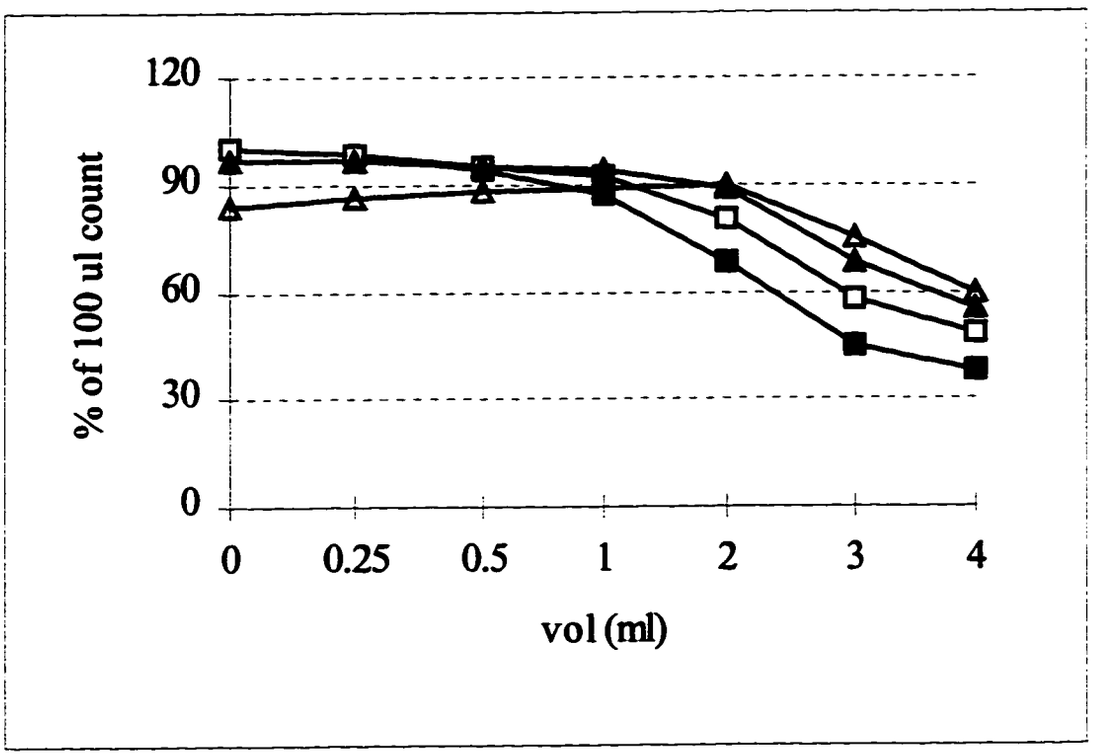

FIGURE 2.1 - ^{125}I - Geometry Correction in Gamma Counter

Volume related differences in counting efficiency in the Cobra gamma counter for varying volumes at elevator positions 1 (■), 2 (□), 3 (▲), and 4 (Δ). Equal number of counts (cpm) were diluted from .25 to 4 mls and counted at the available elevator positions 1 to 4.

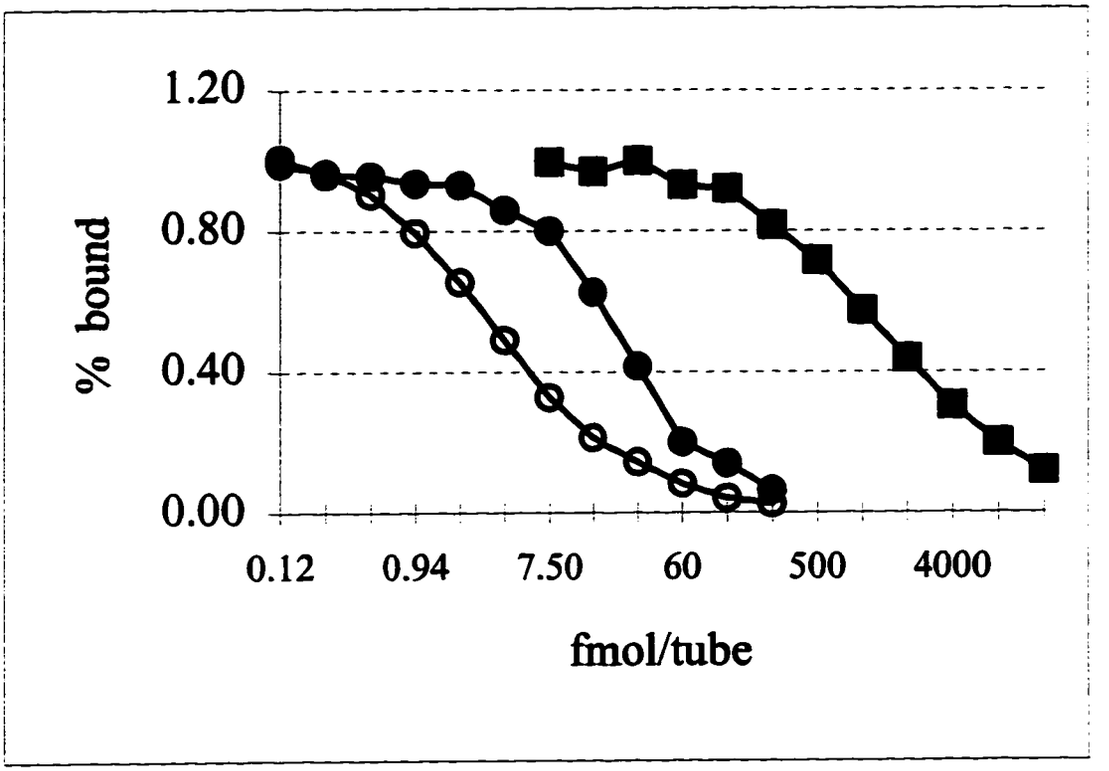

FIGURE 2.2 - Inhibition Curves for CCK-8s, CCK-33, and CCK-8ns

Serial dilutions of standard CCK-8s (●), CCK-33 (○), and CCK-8ns (■) ranging from 0.12 to 240 fmol/tube for CCK-8s and CCK-33, and 7.8 to 16,000 fmol/tube for CCK-8ns.

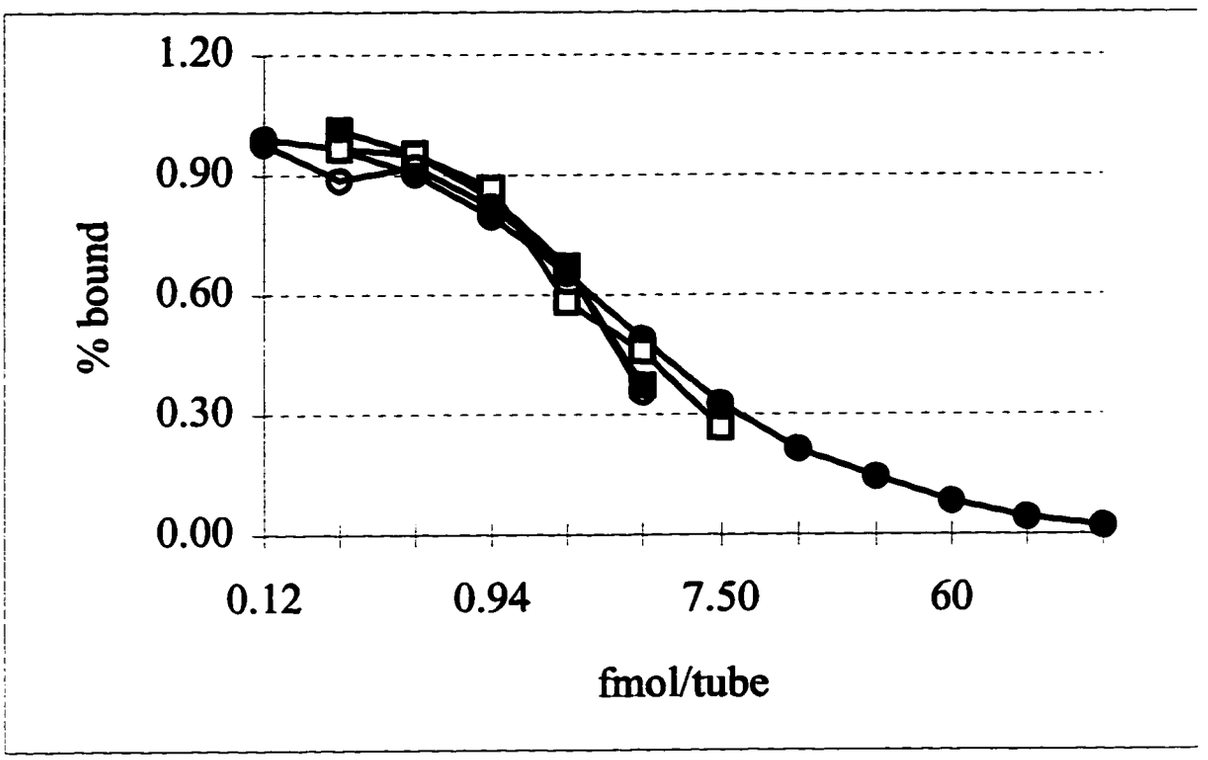

FIGURE 2.3 - Parallelism Results after Sep-Pak Extraction

Serial dilutions of three differently discoloured samples after Sep-Pak extraction compared to CCK-8s inhibition curve (●). Dark (○), medium (▫), and light (▪) discoloured sample.

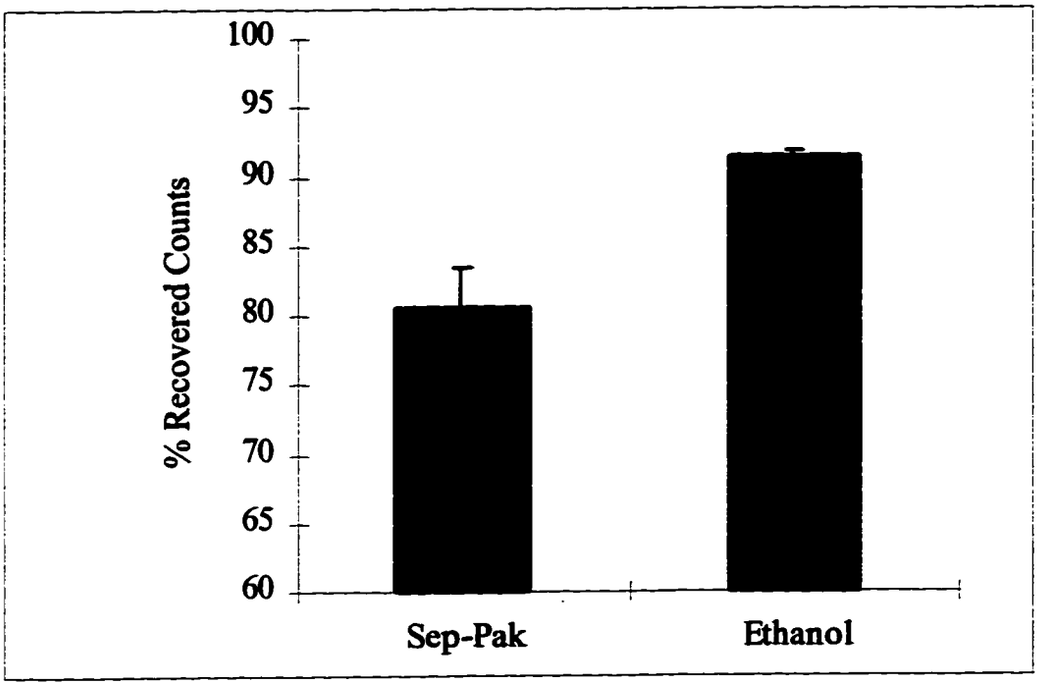

FIGURE 2.4 - Percentage Efficiency of Extraction of ^{125} I-CCK from Plasma

Extraction efficiency of Sep-Pak or ethanol extraction estimated after addition of ^{125}I-CCK to plasma by measuring the percentage of plasma counts recovered. Bars: means; error bars: SEM; n=23 for Sep-Pak-, n=18 for Ethanol-Extraction.

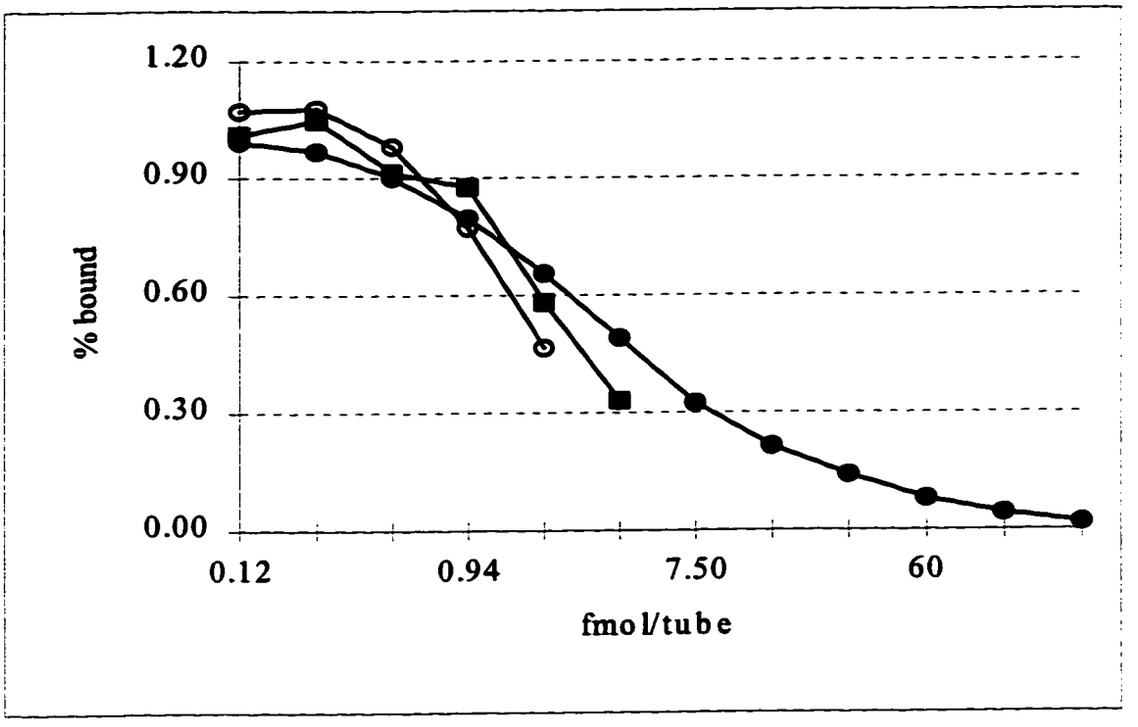

FIGURE 2.5 - Parallelism Results after Ethanol Extraction

Serial dilutions of two samples after Ethanol extraction compared to CCK-8s inhibition curve (●). Internal standard sample (■), and other (○) sample.

2.7 Literature Cited

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Chapter 3 - Development of an In Vivo Method to Study Bovine Intestinal Response to Dietary Manipulation at the Molecular Level[1]

3.1 Introduction

Cells in the gastrointestinal tract respond to changes in nutrient supply as part of an integral feedback system that ensures adequate intake for maintenance, lactation and growth. Although their primary function is considered to be nutrient absorption, the cells also have key roles in the endocrine and neural network. For example, nutrients reaching the small intestine induce the release of gastrointestinal hormones such as cholecystokinin (CCK) which regulate pancreatic secretion, gallbladder contraction, gut motility, serve as satiety signals and likely have other as yet unknown functions (Rehfeld et al., 1986). In the past, animals had to be sacrificed in order to access gastrointestinal tissues (cells) needed to evaluate gene expression and its responsiveness to the presence and characteristics of different nutrients. Increases in the sensitivity of methods for analyzing gene expression (e.g., Northern hybridization analysis) now allows for the use of minute amounts of RNA. Intestinal cannulation of cows together with fiberoptic endoscopy allows easy access to intestinal mucosa without having to sacrifice the animal. Sterzing et al. (1971) also used a biopsy technique to study lipid absorption in cattle. The endoscope used in this study differed from that of Sterzing et al. (1971) in that the fiberoptic bundles allowed for visual observation of the tissue collection using endoscopy forceps. Although only relatively small biopsies can be obtained by endoscopy, the intestinal mucosa is a tissue with a very large surface and immense regeneration capacity, which allows for frequent sampling. The objective of the present study was to develop an intestinal biopsy technique to study nutrient-gene interactions. As part of the validation of the technique the expression of the CCK gene in bovine tissue was studied, CCK mRNA

[1] A version of this chapter has been accepted to be published in the Journal of Animal Science

abundance in biopsy tissues compared with tissues collected postmortem, and changes in CCK mRNA abundance and circulating CCK during nutritional manipulation were investigated.

3.2 Materials and Methods

3.2.1 *Animals, Design, and Sampling Procedures*

The surgical procedures used and housing conditions of the cows met conditions in the "Animals Used for Research or Teaching" protocol approved by the Animal Policy and Welfare Committee at the University of Alberta and were consistent with those of the Canadian Council of Animal Care.

Postmortem Tissue Experiment at Abattoir. Duodenal tissue was collected after slaughter at a local abattoir from 10 Holstein cows from the University of Alberta dairy herd. Prior to slaughter three non-lactating and four lactating cows were fed total mixed rations (TMR) balanced to meet the stated nutrient requirements (NRC, 1989). The other lactating animals were deprived of feed for 3 d before slaughter. On each of 3 d, one animal from each group was shipped to the abattoir, on the first day the additional lactating fed animal was also shipped (Alsask Beef Company, Edmonton, Alberta, Canada). The gastrointestinal system was removed approximately 40 min after the animal's death and intestinal segments were separated, rinsed with ice cold phosphate buffered saline (PBS, pH 7.0), and mucosal tissue was collected from the intestinal lumen by scraping with sterile glass microscope slides on sterile foil covered trays. Mucosa was taken from duodenum, jejunum, and ileum; tissue samples were also collected from the rumen. Tissue and mucosa samples were immediately placed into polypropylene containers, frozen in liquid nitrogen, and stored at -80°C until RNA analysis. The time from the removal of the tissues from the carcass to the final freezing was on average 10 min for the gastrointestinal tissues. Blood samples (25 ml) were taken from each cow twice; three days prior to and on the morning of shipment to the abattoir. Blood was placed into 3 ice-cold Vacutainers (containing 5 μmol EDTA/ ml blood) and plasma was

separated within 30 min of collection. The plasma was aspirated, transferred into a polystyrene tube, and stored at -25 °C until used in the radioimmunoassay.

Postmortem Tissue Experiment at Research Facilities. To demonstrate the longitudinal distribution of CCK gene expression in the bovine gastrointestinal tract mucosa (abomasum, duodenum, jejunum, and ileum) and tissue (rumen and omasum) samples were taken from two lactating dairy cows, pooled, and snap frozen as described above within 30 min of the animal's death. These animals were sacrificed at the research facility to avoid any potential stress associated with transportation.

Biopsy Tissue Experiment. Intestinal biopsies were taken from three multiparous early lactation Holstein cows (650 kg BW) surgically fitted with ruminal (10 cm i.d., Bar Diamond Inc., Parma, ID), and T-type duodenal and ileal cannulae as described previously (Robinson et al., 1990). After the surgery the cows were housed in tie stalls with access to water at all times and fed a TMR once a day at 0900 balanced to meet the stated nutrient requirements (NRC, 1989). The amount of lipid reaching the small intestine was manipulated using an abomasal infusion technique described previously (Drackley et al. 1992). The infusion lines were inserted into the omasal-abomasal orifice 6 h prior to the start of the experiment. Feed was withheld for 4 h prior to the start of the experiment and was made available at the start of the infusion. During a 4 h period, 0 (saline infusion), 80.1, or 188.7 g of canola oil was infused, which was equivalent to 1.5, 3.3, and 5.1%, respectively, added lipid in the TMR. The saline infusion was equivalent to 1.5% added lipid because the concentrate used in the TMR contained lipid.

A fiberoptic endoscope (Olympus CF type 1B) and biopsy forceps were used to obtain duodenal tissue samples (10 to 25 mg) approximately 40 to 50 cm distal from the pyloric sphincter. The biopsies were taken while the cows were in their tie stalls. The first step in the biopsy procedure was the removal of the plug of the t-type cannula, thus allowing the digesta coming from the abomasum to drain out of the cannula. As a result, the distal part of the duodenum becomes relatively clean, facilitating the biopsy procedure. The procedure involved two people; one to look through and operate the endoscope and the other to advance or retract the flexible end as instructed by the

operator. When there was a clear view of the biopsy site the biopsy forceps were fed into the appropriate channel in the endoscope. After the forceps appeared in the field of view of the operator they were opened, guided towards the intestinal mucosa layer, closed, and retrieved. Two biopsies samples were taken (approximately 2 to 8 cm apart) from each animal 1 h prior to, and .5, 1, 2, 4, and 24 h after the start of the infusion. The biopsies were rinsed in cold PBS, placed into a sterile microcentrifuge tube, frozen in liquid nitrogen, and stored at -80°C until RNA isolation and analysis.

3.2.2 *Assays*

RNA isolation, Gel Electrophoresis, and Northern Transfer. From .9 to 1 g of postmortem intestinal tissue was used for RNA isolation using a standard GIT-CsCl procedure as described by Glimm et al. (1992). Polyadenylated RNA (poly A^+ RNA) was isolated from duodenal total RNA using LiCl based buffers and oligo(dT)-cellulose chromatography (Glimm et al., 1992). RNA depleted of polyadenylated RNA (poly A^- RNA) was used as a negative control and poly A^+ RNA as well as total RNA isolated from rat duodenal mucosa were used as positive controls in each Northern hybridization.

Two methods of isolating RNA from the test biopsies were evaluated. The first method was a down-scaled GIT-CsCl method that used a Beckmann Airfuge (Rappolee et al., 1989). The second method was a small scale version of the TRIzol® method (Gibco, Life Technologies, Burlington, ON, Canada). Although both methods gave comparable results in subsequent Northern hybridization analysis, we chose the easier TRIzol method for isolating RNA in the work reported herein. The TRIzol method involved manually homogenizing the biopsies for 1 min with 50 μL of TRIzol in a 1.5 ml polypropylene microcentrifuge tube using a microcentrifuge pestle (Kontes, Vineland, NJ. USA). After 30 s of centrifugation (12,000 *g*) the sample was again homogenized for 30 s, 450 µL of TRIzol were added, and the sample was homogenized for another 30 s. Homogenized samples were kept on ice until all homogenization was complete. After a 5 min incubation at 22°C, 100 µL of chloroform were added, the sample vigorously mixed for 15 s and incubated at 22°C for another 5 min. The sample was then centrifuged

(12,000 *g*) for 20 min at 4°C and the supernatant was transferred into a new 1.5 ml polypropylene microcentrifuge tube, mixed with an equal volume of isopropanol, and precipitated overnight at -20°C. The sample was extracted using phenol-chloroform-isoamylalcohol (25:24:1, vol/vol/vol) and then extracted using chloroform-isoamylalcohol (24:1, vol/vol). The RNA was precipitated by the addition of 2.5 volume 95% ethanol (vol/vol) and a one tenth of a volume of 3.5 *M* sodium acetate resuspended in TE buffer, and quantified by UV absorption at 260 nm. The UV light absorbance ratio (260/280 nm) of RNA ranged from 1.89 - 2.03. To determine whether there were differences in RNA abundance in different regions of the duodenum, a second set of biopsies taken at the same time as the first was also used for RNA isolation and analysis. Total RNA (15 μg) from the biopsies and total RNA (40 μg) from the postmortem duodenal tissue were separated by electrophoresis in denaturing agarose gels and transferred to Zeta-probe nylon membranes (Bio-Rad, Canada) as described previously (Glimm et al., 1992). After electrophoresis and transfer, membranes were baked under vacuum at 80 °C for 2 h.

Radiolabeled Riboprobes and Northern Hybridization. A CCK cDNA fragment (Gift from J. Dixon, Purdue University, West Lafayette, IN) was subcloned into the plasmid pBluescript®II SK (Stratagene, PDI-Bio Science, Rockford, IL). A radiolabeled (^{32}P-CTP) CCK antisense riboprobe was generated from HindIII-linearized plasmid DNA using T3 RNA polymerase (Promega, Madison, WI). Template DNA was digested with DNase and the riboprobe purified by phenol-chloroform extraction and Sephadex G-50 (DNA grade fine, Pharmacia, Alameda, CA) chromatography (Glimm et al., 1992). Membranes were prehybridized, hybridized using the CCK riboprobe at 2 x10^6 dpm/ml of hybridization solution, and then washed as described (Glimm et al., 1990) with the most stringent wash being performed at 75°C for 35 min instead of 70°C. Autoradiography of Northern blots was performed for 4 to 72 h at -70 °C using X-ray films (Kodak XAR 5, Eastma Kodak, Rochester, NY, USA) and an intensifier screen (Dupont, Canada, Missisauga, ON, Canada). The audioradiograms were analyzed by imaging densitometer (model GS-670, Bio Rad Laboratories, Ltd, Missisauga, ON,

Canada) and the values for each band were corrected for loading differences based on the intensity of the corresponding Ethidium bromide stained 28S rRNA band on the membrane after completing the transfer as recommended by Bathia et al. (1994).

Radioimmunoassay. Plasma samples were thawed on ice and two aliquots of 1.5 Ml were mixed with 3 Ml 98% ethanol (vol/vol), vortexed for 10 s, and centrifuged at 2000 *g* for 15 min. The supernatant was poured into a 5 Ml glass tube and dried overnight in a rotary evaporator. Details of the CCK RIA are described in Chapter 2.2, 2.4 and 2.5 of this thesis.

Statistical Analysis. To determine differences between the treatments in the postmortem tissue experiment at the abattoir LSD were used to analyze the data for CCK mRNA abundance and plasma CCK concentration. For the plasma data the difference between the CCK plasma concentration before and after the treatment were used. In order to account for the unequal number of animals per group p-diff was used. Computations were done using the GLM procedure of SAS (1985), statistical significance was defined as $p \leq 0.05$.

3.3 Results and Discussion

3.3.1 *Postmortem Tissue Experiments*

CCK mRNA abundance. FIGURE 3.1 shows the results of a preliminary experiment to establish the amount of RNA required to detect a CCK mRNA signal. Tissue collected at the abattoir was used to isolate the bovine RNA for this experiment and there were no signs of degradation observed after gel electrophoresis (visual observation of the 28S and 18S rRNA). The electrophoresis gel was loaded with 10 and 20 μg A^+ RNA from bovine duodenum; 40 μg A^- RNA from bovine duodenum; 10, 20, and 40 μg total RNA from bovine duodenum; 10, 20, and 40 μg total RNA from rat duodenum; 40 μg A^- RNA from rat duodenum; and 10 and 20 μg A^+ RNA from rat duodenum. A strong CCK mRNA signal was detected in rat total RNA as well as in all A^+ RNA samples. Other than a low level of non specific binding of the CCK riboprobe to 28S and 18S ribosomal RNA the

A^- RNA did not show any signal. After a long exposure (26 h), a relatively weak signal of CCK mRNA could be detected in 40 μg total RNA isolated from bovine duodenum (result not shown).

FIGURE 3.2 shows a comparison of the abundance of CCK mRNA in duodenal tissue collected from the ten cows at the local abattoir, and again there were no signs of degradation observed after gel electrophoresis. Only a weak CCK mRNA signal was detected in all of the cows, even after an extended exposure time of approximately 26 h. As a result of the long exposure time, a considerable amount of nonspecific binding to 28S and 18S ribosomal RNA was observed. Large differences in CCK mRNA between cows were observed. The weak signal of CCK and the magnitude of the individual differences together with the small number of animals are likely reasons for no detectable significant differences in CCK mRNA abundance between the fasted and fed cows. In jejunum and ileum collected postmortem, a CCK mRNA signal was also detectable in 40 μg total RNA though at a lower level than in duodenal tissue (results not shown).

In rumen tissue collected postmortem CCK mRNA was detected in 20 μg A^+ RNA but not in 40 μg total RNA (FIGURE 3.3). In contrast to the situation in the intestine, the mucosa of the rumen could not be scraped off due to morphological structure. The postmortem rumen samples, therefore, represent an entire cross section of the rumen wall, which contains many different cell types (e.g. muscle, neuronal, endothelial). Considering that intravenously administered CCK has been shown to affect rumen motility (Grovum et al., 1981) it is conceivable that the CCK mRNA we detected in rumen tissue is derived from neuronal cells. FIGURE 3.4 shows the longitudinal distribution of CCK gene expression in the bovine gastrointestinal tract. The tissues used for RNA isolation were derived from two animals that were slaughtered at our research facility. It can be seen that in duodenal, jejunal, and ileal scrapings CCK mRNA can be visualized in 5 μg A^+ RNA and even in 30 μg total RNA.

3.3.2 *Biopsy Collection Experiment*

Using a CCK antisense riboprobe in the Northern hybridization technique a

relatively strong signal was produced by 15 μg total RNA isolated from biopsies after an 8 h exposure of the autoradiogram (FIGURE 3.5). Equally intense signals were observed with two positive controls; 15 μg total RNA isolated from rat duodenum and 15 μg A^+ RNA isolated from bovine duodenal tissue obtained postmortem. In both the negative control, which was 15 μg A^- RNA, and in 40 μg total RNA isolated from postmortem duodenum no signal was detected after 8 h exposure. Cholecystokinin mRNA abundance increased during the oil infusion, reaching its highest value after 2 h for the 3.3 % added lipid treatment and after 1 h for the 5.1% added lipid treatment. No significant increase in CCK mRNA abundance could be detected during the saline infusion. Differences in CCK mRNA abundance between animals were apparent in the samples taken before infusion. However, the relative differences were maintained over the time course of biopsies, which allows relative changes induced by infusion to be measured. The autoradiogram depicting the results of the infusion is shown in FIGURE 3.5A. Longer exposures of the autoradiogram (up to 64 h) revealed nonspecific binding of the CCK riboprobe to 28S and 18S ribosomal RNA. Only a weak CCK mRNA signal was detected in 40 μg total RNA isolated from postmortem duodenum; a weak signal from nonspecific binding of the CCK riboprobe to 28S and 18S ribosomal RNA was also observed on these blots. FIGURE 3.5B shows the results obtained using the second set of biopsies that were taken during the same experiment with rat total RNA as a positive control and bovine A^- RNA as a negative control. Comparing the signal intensities in FIGURE 3.5A and FIGURE 3.5B, it appears that there are small local differences in CCK mRNA abundance within the bovine duodenum as the biopsies were taken approximately 2 to 8 cm apart. This difference is likely attributable to one or both of two things. The CCK gene is known to be expressed in typical endocrine cells (I cells) of the small intestine. Because those cells may be distributed in varying density over the duodenal lumen it is possible that there are small local differences in CCK mRNA. However the intestinal tissue biopsies vary in size and consequently also in the amount of tissue layer combination from mucosa and the underlying connective tissue and muscle layers. We have not studied the histological distribution of these layers in intestinal biopsies of different sizes, however Sterzing et al. (1971) found that the intestinal biopsies collected with a hydraulically operated suction

biopsy tube from intestinally cannulated calves were separated from the intestine at the level of the muscularis mucosae, thus indicating the biopsy to be a mixture of different tissues. The suction biopsy tube used by Sterzing et al. (1971) allows for larger biopsy samples to be excised but does not allow for visual observation of the sampling procedure due to lack of fiberoptic fibers, which reduces the safety of the procedure. For future experiments, we recommend taking a minimum of three biopsies, which should be pooled prior to RNA isolation, to give a representative mean of the CCK mRNA abundance present in the bovine duodenum.

There was a marked difference in the abundance of CCK mRNA in duodenal tissue obtained by biopsy compared to that collected postmortem at the abattoir. These findings are in agreement with the observation of Sterzing et al. (1971) who noted that after electron microscopy of intestinal biopsy tissue, intact epithelium was observed which was not the case for slaughtered animals. During our tissue collection at the abattoir the animal carcasses were on the processing line for a minimum of 40 min prior to our having access to the gastrointestinal tissue, whereas slaughtering at our research facility allowed for completed tissue collection within 20 to 30 min of the animal's death. Although it seems possible that CCK mRNA degradation could be the reason for low CCK mRNA abundance in the tissue collected at the abattoir, visible RNA (28S and 18S rRNA) obtained from both biopsies and postmortem tissue was observed to be intact showing no sign of degradation during the Northern hybridization procedure. It is likely that the duration of slaughter had no apparent effect on CCK mRNA abundance even though we did not measure CCK mRNA half life. It is very likely that stressors (e.g., shipment, unfamiliar environment and handlers) associated with slaughter at the abattoir contributed to the lower abundance of CCK mRNA observed in all postmortem tissue samples. Tissues that were collected after slaughter at the research facility (animals not transported to the abattoir) show a relatively higher CCK mRNA abundance (FIGURE 3.4). These findings support the hypothesis that stressors on the animal were responsible for lower CCK mRNA abundance even though we did not systematically test the effect of time from the death of the animal to tissue freezing on CCK mRNA. The endoscopy

biopsy technique avoided such stressors because it was performed in the cow's familiar environment (i.e., tie stall) by familiar handlers and also allowed freezing of tissue samples nearly immediately after biopsy separation from the intestine.

3.3.3 *CCK Blood Concentration:*

In spite of storage of the frozen blood plasma samples at -25°C for nearly 2 years before performing the CCK radioimmunoassay, CCK blood concentration was within the normal range (3.8 to 25.6 pmol l^{-1} compared to fresh plasma samples that were analyzed during later experiments. FIGURE 3.6 shows the blood concentrations of CCK three days before and on the morning of shipment to the abattoir. At the time of blood sampling the cows were still in their accustomed environment. Other than the fasting of one of the groups there was relatively little stress imposed on the animals. It has been shown in non-ruminants that CCK regulates gall bladder contraction (Ivy et al., 1928) and pancreatic enzyme secretion in response to nutrients reaching the small intestine (Reidelberger et al., 1986; Rosewicz et al., 1989). It is also believed that CCK is a satiety hormone for short term feed intake control in rats, pigs, and humans (Gibbs et al., 1973; Gregory et al., 1989; Lieverse et al., 1994). Thus, based on existing information it was expected that the fasted cows would have a lower blood concentration of CCK after feed deprivation. With less nutrients reaching the small intestine there should be less stimulation for CCK gene expression and CCK synthesis and release. Our findings are therefore consistent with current knowledge because there was a significant decrease in the blood concentration of CCK after 3 days of fasting. On the other hand a significant decrease in CCK mRNA abundance in the mucosa of the small intestine was not observed, possibly due to the stressors on the animals during shipment which occurred after blood collection. It therefore remains to be determined whether fasting influences transcription and RNA turnover of the CCK gene and thereby CCK mRNA abundance in cells in the bovine small intestine. Based on our findings, additional research with more animals and fewer stressors is required. The intestinal biopsy technique described in this report seems ideally suited for rapid collection of intestinal tissue for the study of nutrient-gene interaction.

3.4 Implications

Experiments were conducted to evaluate the effectiveness of an intestinal biopsy sampling method to study nutrient-gene interactions. That evaluation revealed the usefulness of the method for studying questions about the influence of nutrients reaching the small intestine on the expression of gastrointestinal genes and possible implications for feed intake regulation. It was also shown that the biopsy sampling method is superior to collecting intestinal tissue after slaughter in an abattoir because of reduced stress and reduced time from sampling to snap-freezing of the tissue. The use of the biopsy technique is not limited to monitoring responses to dietary manipulation. It should also prove useful in absorption studies, as well as evaluating a wide spectrum of gastrointestinal changes such as those occurring during lactation and metabolic disorders.

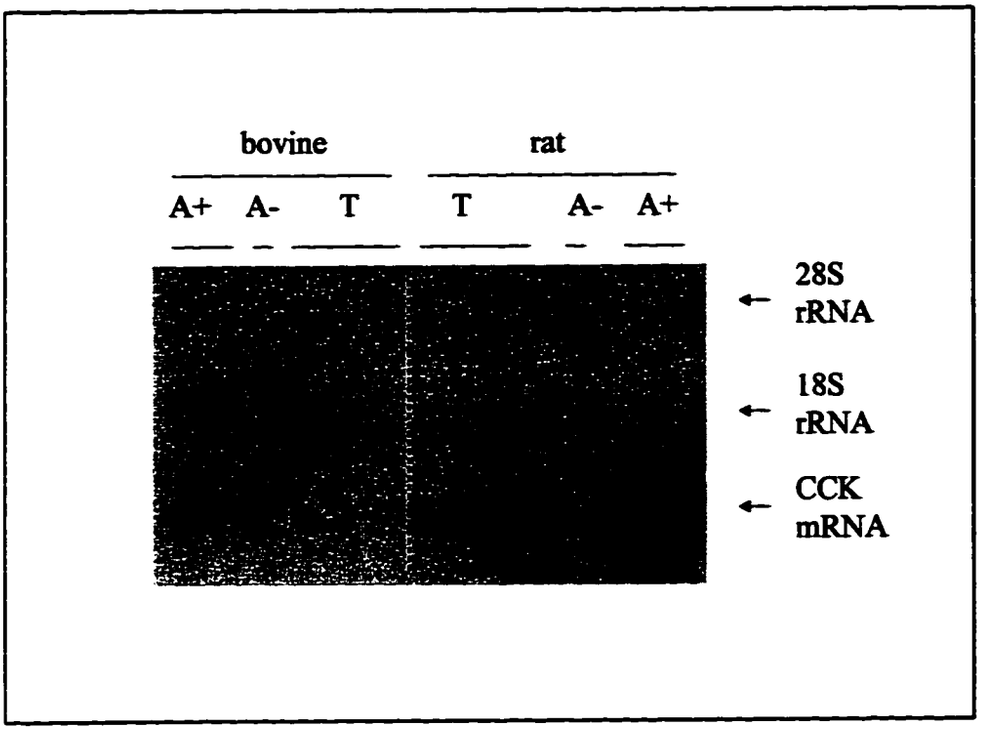

FIGURE 3.1 - CCK mRNA Abundance in Duodenal Mucosa of Bovine and Rat

Autoradiogram depicting cholecystokinin (CCK) mRNA abundance in duodenal mucosa of cows and rats. Different amounts of bovine and rat total RNA (T) (10, 20, and 40 μg), bovine and rat polyadenylated RNA (A^+) (10 and 20 μg), and bovine RNA depleted of polyadenylated RNA (A^-) (40 μg) were separated by electrophoresis and probed for CCK mRNA using the technique of Northern hybridization. The location of 28S and 18S ribosomal RNA and the CCK mRNA band are indicated. Autoradiographic exposure was 5.5 h.

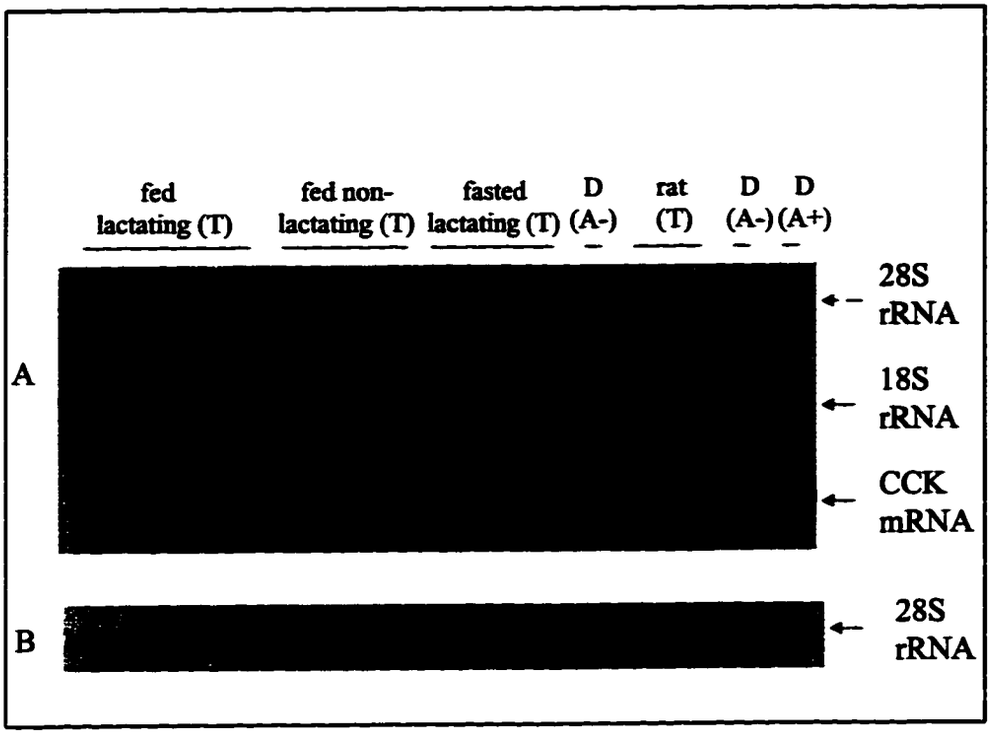

FIGURE 3.2 - Effect of Fasting, Feeding and Stage of Lactation on CCK mRNA Abundance in Bovine Duodenal Mucosa (Postmortem)

Autoradiogram depicting the effect of fasting, feeding, and stage of lactation on cholecystokinin (CCK) mRNA abundance in bovine duodenal mucosa collected postmortem at an abattoir (A). Ten Holstein cows were fed to their requirements or fasted for 72 h. Forty micrograms bovine or rat duodenal total RNA (T), 40 μg bovine duodenal RNA depleted of polyadenylated RNA (D(A^-)) and 10 μg bovine duodenal poly-adenylated RNA (D(A^+)) are shown. The location of 28S and 18S ribosomal RNA and the CCK mRNA band are indicated. A photograph of the Northern transfer membrane is also presented (B). Ethidium bromide stained 28S ribosomal RNA reveals equal loading of similar amounts of RNA in different lanes. Autoradiographic exposure was 26 h.

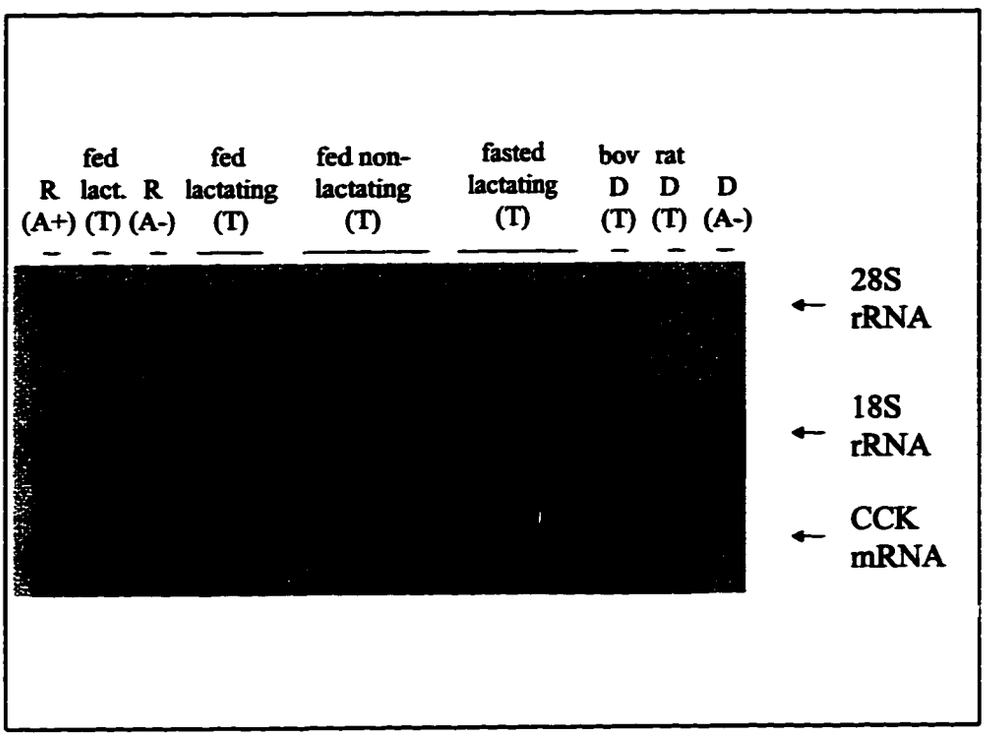

FIGURE 3.3 - Effect of Fasting, Feeding and Stage of Lactation on CCK mRNA Abundance in Bovine Rumen Tissue (Postmortem)

Autoradiogram depicting the effect of fasting, feeding, and stage of lactation on cholecystokinin (CCK) mRNA abundance in bovine rumen tissue collected postmortem at an abattoir. Nine Holstein cows were fed to their requirements or fasted for 72 h. Forty micrograms of bovine rumen total RNA (T), bovine duodenum total RNA (bov D (T)), 20 μg rat duodenum total RNA (rat D (T)), 40 μg bovine rumen RNA depleted of polyadenylated RNA (R (A^-)), 40 μg bovine duodenum RNA depleted of polyadenylated RNA (D (A^-)), and 20 μg bovine rumen polyadenylated RNA (R (A^+)) are shown. The location of 28S and 18S ribosomal RNA and the CCK mRNA band are indicated. Autoradiographic exposure was 26 h.

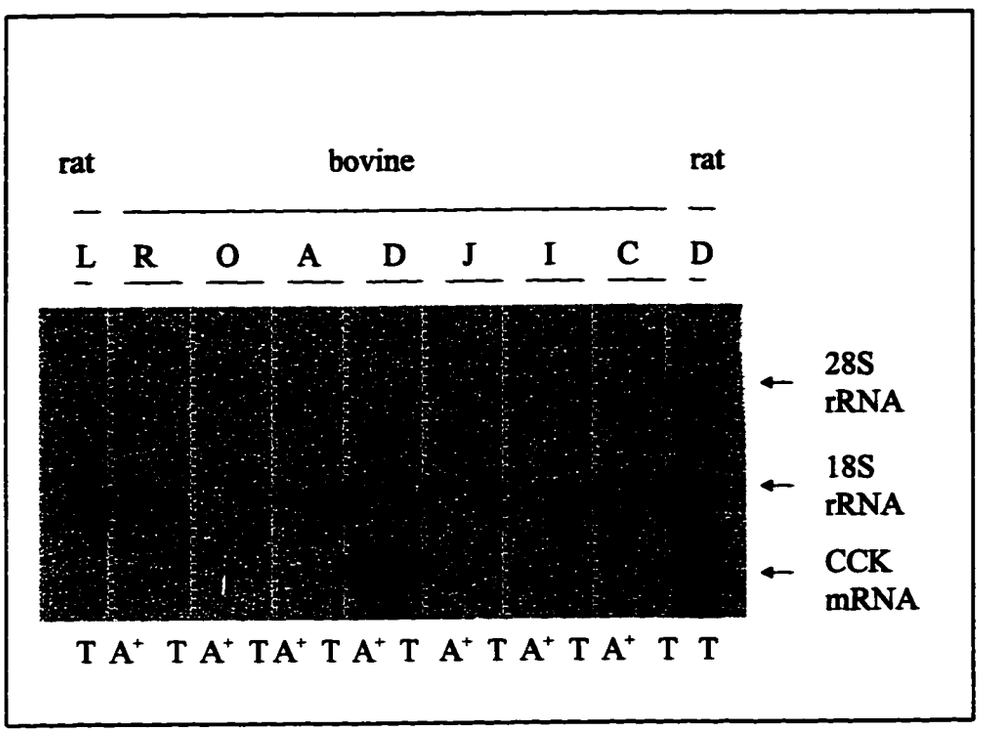

FIGURE 3.4 - Longitudinal Distribution of (CCK) mRNA in the Bovine Gastrointestinal Tract.

Autoradiogram depicting the longitudinal distribution of cholecystokinin (CCK) mRNA in the bovine gastrointestinal tract. Tissues were collected postmortem at the University of Alberta research facility. Thirty microgram of total RNA (T) and 5 μg polyadenylated RNA (A^+) of bovine rumen (R), omasum (O), abomasum (A), duodenum (D), ileum (I), jejunum (J), and caecum (C) as well as 30 μg rat liver (L) and rat duodenum (D) are shown. Autoradiographic exposure was 16 h.

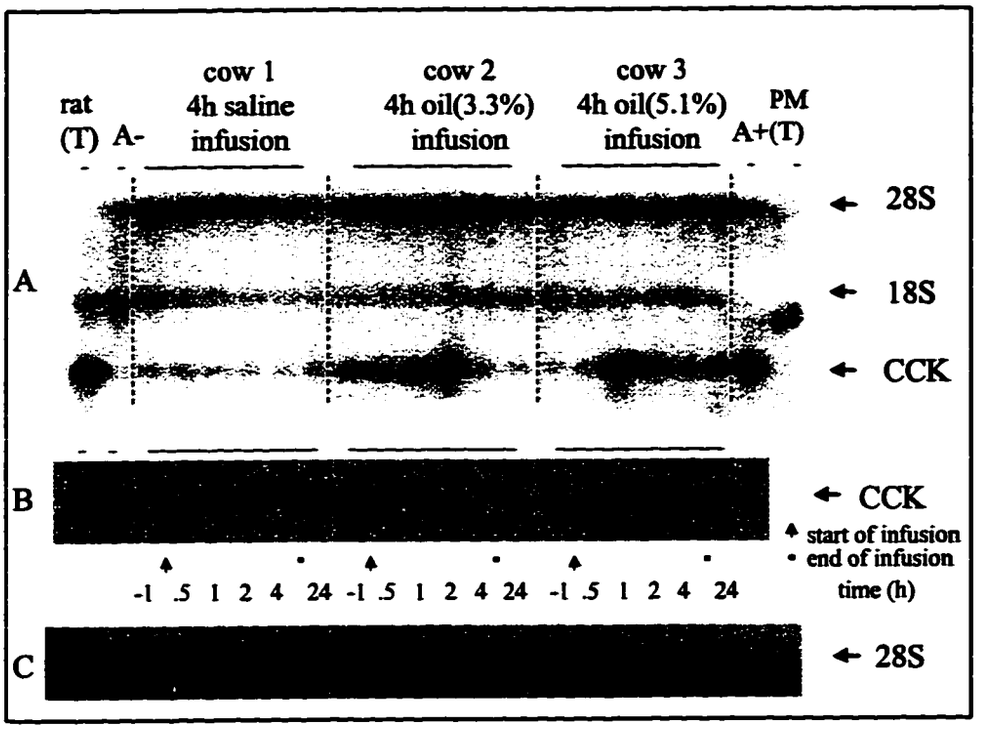

FIGURE 3.5 - Effect of Abomasal Infusion of Canola Oil on CCK mRNA Abundance in Bovine Duodenal Mucosa (Biopsy Preliminary Study)

Autoradiogram depicting the effect of abomasal infusion of canola oil on the duodenal cholecystokinin mRNA abundance in cows. For comparison, two autoradiograms are presented (A and B), each derived from one of a set of two biopsies taken 2 to 8 cm apart in the duodenum during the same experiment from the same animals. In a 15 μg of duodenum total RNA isolated from the first set of biopsies, 15 μg rat duodenum total RNA (rat (T)), 15 μg bovine RNA depleted of polyadenylated RNA (A^-), 15 μg bovine polyadenylated RNA (A^+), and 40 μg bovine duodenum total RNA collected postmortem (PM (T)) are shown. The location of 28S and 18S ribosomal RNA and the CCK mRNA band are indicated. In b, the CCK mRNA band of fifteen microgram of duodenum total RNA isolated from the second set of biopsies, 15μg rat duodenum total RNA (rat(T)), and 15 μg bovine RNA depleted of polyadenylated RNA (A^-) are shown. Both autoradiograms were exposed for 5 to 6 h. A photograph of the Northern transfer membrane is also presented (C). Ethidium bromide stained 28S ribosomal RNA again reveals equal loading of similar amounts of RNA in different lanes.

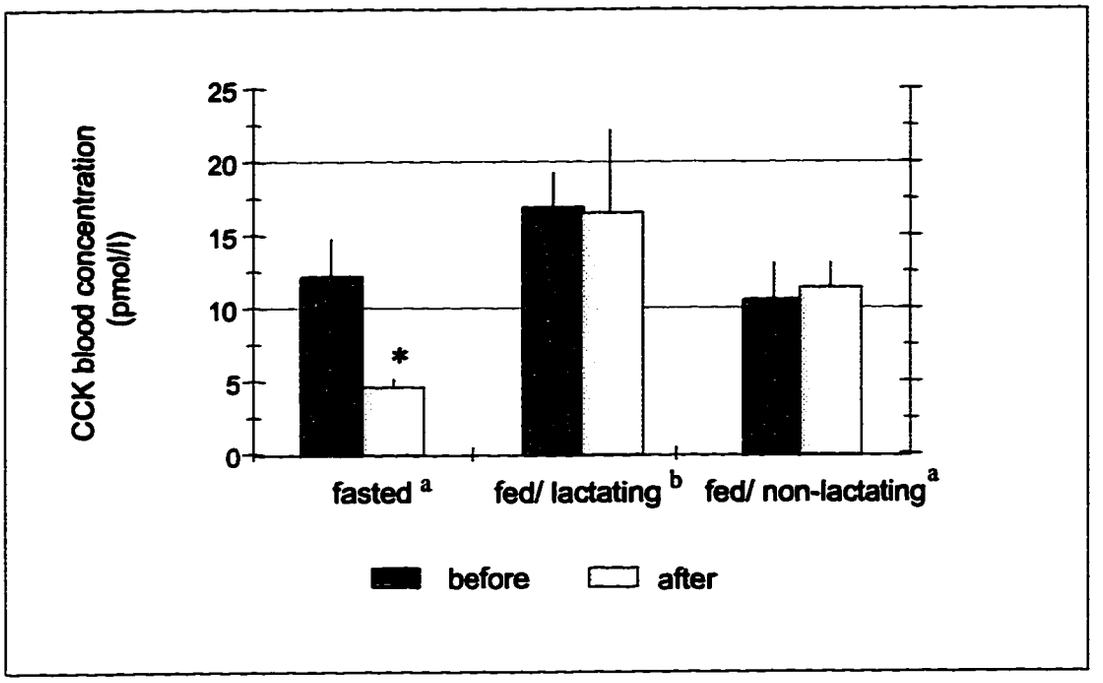

FIGURE 3.6 Effect of Feed Restriction and Physiological Status on Blood Concentration of CCK in Dairy Cattle

Blood concentration of CCK in pmol l^{-1} before and after a three day period of fasting or feeding to meet NRC requirements.

[a] n=3; [b] n=4; * 'after' values significantly different from 'before' values $p \leq .05$.

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Chapter 4 - Intestinal Nutrient-Gene Interaction: Effect of Fasting and Refeeding on Cholecystokinin and Proglucagon Gene Expression[2]

4.1 Introduction

Cholecystokinin (CCK) is a neuropeptide found primarily in I-cells in the proximal small intestine (Buffa et al., 1976) and in the brain (Dockray, 1976). It is released in response to food ingestion (Rehfeld, 1986). Functions of CCK of intestinal origin include gallbladder contraction (Ivy and Oldberg, 1928) and pancreatic digestive enzyme secretion (Reidelberger et al., 1986; Rosewicz et al., 1989), but it has also been associated with eating disorders (Geracioti and Liddle, 1988). A role for CCK as a satiety factor has been studied in several species including rats (Gibbs et al., 1973), pigs (Anika et al., 1981; Gregory et al., 1989), sheep (Della-Fera and Baile, 1980) and humans (Kissileff et al., 1981; Lieverse et al., 1994). Dietary regulation of intestinal CCK gene expression has been demonstrated in rats (Liddle et al., 1988) and differential brain and gut CCK gene expression in fasted rats has also been shown (Zheng et al., 1987; Greenstein et al., 1990).

Glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) are the major intestinal proglucagon product. Secreted from the L-cells predominantly in the distal ileum (Ørskov et al., 1986), GLP-1 effects in monogastric species are insulinotropic - when glucose plasma concentrations are elevated (Holst et al., 1987) - and glucagonostatic (Komatsu et al., 1989). Inhibition of gastric acid secretion (O'Halloran et al., 1990) and gastric emptying (Wettergren et al., 1994) have also been demonstrated. A role for GLP-1 in the central regulation of feeding has also been suggested even though peripherally administered GLP-1 was reported not to affect feed

[2] A version on this chapter was presented in abstract form at the American Dairy Science Association annual meeting in Guelph, Ontario, Canada, June 1997, J. Dairy Sci. 80(1) P167.

intake (Turton et al., 1996). Proglucagon mRNA abundance and GLP-1 release was shown to decrease during fasting and increase after refeeding in rats (Hoyt et al. 1996). In sheep, GLP-1 has been shown to induce an increase in blood insulin concentration during hyperglycaemic conditions comparable to that induced by postprandial blood glucose (Martin and Faulkner, 1993). GLP-1 plasma concentrations in sheep have been reported to be 15 to 25 pmol l^{-1} by Martin and Faulkner (1996).

Traditionally, feed intake regulation in ruminants is believed to occur primarily at the level of the first stomach. Rumen fill, passage rate of digesta through the forestomachs, and accumulation of ruminal fermentation products are thought to be the main factors controlling feed intake in ruminants (Baile and Della-Fera, 1988). Interest in intestinal feed back mechanisms regulating feed intake in monogastrics has only recently commenced in ruminants. As a result information regarding factors regulating CCK and GLP-1 in ruminants are limited. However, if CCK and GLP-1 were shown to be released in response to nutrients reaching the intestine in ruminants then they would comply with one of five criteria established by Smith (1984) for physiological satiety effect of a gut hormone and thus support the hypothesis that feedback from nutrients in the intestine play a role in feed intake regulation in ruminants. To explore this possibility we investigated the hypothesis of dietary regulation of CCK and proglucagon gene expression as well as CCK and GLP-1 release in the bovine small intestine during a period of fasting and refeeding.

4.2 Materials and Methods

Animals and sampling protocol. The experiment was carried using five female cattle (550 ± 50 kg BW) surgically fitted with ruminal (10 cm i.d., Bar Diamond Inc., Parma, ID), duodenal, and ileal cannulae as described previously (Robinson and Kennelly, 1990). The animals were allowed to recover from surgery for a minimum of two months. Prior to the experiment the animals were fitted with jugular catheters (micro-renethane, Braintree Scientific; .04 inch i.d.). The animals were housed in tie stalls with access to water at all times and were accustomed to once a day feeding (0900 h) of a total

mixed ration balanced according to NRC (1989) requirements. The amount of feed consumed was continuously recorded with automatic feed scales.

A fiberoptic endoscope (Olympus CF type 1B) and biopsy forceps (type 24 F, Carson Group, Toronto, ON, Canada,) were used to obtain duodenal and ileal tissue samples (10 to 25 mg) approximately 40 to 50 cm distal to the abomasal sphincter and 30 cm proximal to the caecal-ileal orifice. Each biopsy sampling consisted of three duodenal and two ileal biopsies taken approximately 3 to 5 cm apart. The biopsies were rinsed in PBS (pH 7.4), placed in a sterile microcentrifuge tube, frozen in liquid nitrogen, and stored at -80°C until RNA isolation and analysis. Blood (8 ml) was placed in an ice-cold Vacutainer containing 5 μmol ml^{-1} blood EDTA, or 10 μmol ml^{-1} blood EDTA and 500 KIU ml^{-1} Aprotinin (Trasylol, Bayer, Etobicoe, ON, Canada) for the CCK and GLP-1 radioimmunoassay (RIA) respectively, stored on ice, and plasma separated within 30 min of collection. Plasma from each blood sample was stored at -70°C until ethanol extraction before RIA.

Experimental design. Before the start of the fasting period (-12 h) intestinal biopsies and blood samples were taken for baseline values. Feed was removed (0900 h) the following morning and for the next two days was withheld, but access to water was maintained. At 12, 24, and 48 h during the fasting period intestinal biopsies and blood samples were taken. On the third day of fasting (0900 h), the animals were presented the same amount of food that they had consumed the day before fasting and biopsies and blood samples were taken at 2 (blood only), 4, 8, 16, 24, and 36 h after refeeding. Cumulative feed intake was measured three days prior to fasting, and on the first and second day of refeeding at 4, 8, 12, 16, 20, and 24 h after feeding.

Isolation of RNA, gel-electrophoresis, and Northern transfer. Total RNA was isolated from the intestinal biopsies with the TRIzol® method (Gibco Life Technologies, Burlington, ON, Canada). The two ileal or three duodenal pooled biopsies collected at each sampling time were homogenized with TRIzol reagent in a 1.5 ml polypropylene microcentrifuge tube using a microcentrifuge pestle (Kontes, Vineland, NJ, USA)

attached to an upright drill and the RNA isolation completed as described in Chapter 3.2.2. of this thesis. The RNA was resuspended in TE buffer and quantified by UV absorption at 260 nm. The UV absorbance ratio (260/280 nm) of RNA ranged from 1.89 - 2.03.

Total RNA was isolated from intestinal tissue using a standard GIT-CsCl procedure as described previously (Glimm et al., 1992). Polyadenylated RNA (poly A^+ RNA) was isolated from duodenal total RNA as described previously (Glimm et al., 1992) using LiCl buffer and oligo(dT)-cellulose chromatography. Twenty micrograms of RNA depleted of polyadenylated RNA (poly A^- RNA) was used as a negative control. Twenty micrograms of total RNA isolated from rat duodenal mucosa or 15 μg total RNA isolated from rat ileal mucosa was used as positive controls in each of the Northern hybridizations probed for CCK and proglucagon respectively to allow comparison between membranes.

Twenty micrograms of total RNA from the duodenal biopsy samples or 15 μg of total RNA from the ileal biopsy samples of two animals and the positive and negative control were separated by electrophoresis in denaturing agarose gels and the RNA transferred to Zeta-probe nylon membranes (Life Science, Bio-Rad, Mississauga, ON, Canada) as described previously (Glimm et al., 1992). The membranes were baked under vacuum at 80°C for 2 h.

Radiolabeled riboprobes and Northern hybridization. A radiolabeled (^{32}P-CTP) CCK antisense riboprobe was generated as described in Chapter 3.2.2. of this thesis. A proglucagon antisense riboprobe was generated from *Rsa*I linerized pGEM-4 plasmid containing a 440 bp proglucagon fragment (Gift from Dr. P.J. Fuller. Prince Henry's Institute of Medical Research, Clayton, Australia) using SP6 RNApolymerase (Promega). Template DNA was digested with DNase (Promega) and the riboprobe purified by phenol-chloroform extraction and Sephadex G-50 (DNA grade fine, Pharmacia, Baie D'Urfe, QE, Canada) chromatography as described previously (Glimm et al., 1992). Membranes were prehybridized and then those with duodenal RNA hybridized using the CCK riboprobe and those with ileal RNA were hybridized using the proglucagon

riboprobe. Both probes were used at 2 x10^6 dpm ml^{-1} of hybridization solution. After hybridization the membranes were washed as described (Glimm et al., 1990). For the CCK riboprobe hybridized blots, the most stringent wash was performed at 72°C for 30 min followed by 75°C for 5 min. For the proglucagon riboprobe the best results were achieved using a temperature of 70°C for 15 min in the most stringent wash. Autoradiography of Northern blots was performed and analyzed by imaging Densitometer as described in Chapter 3.2.2. of this thesis. The values for each band were corrected for loading differences based on the intensity of the corresponding Ethidium bromide-stained 28S rRNA band on the membrane after completed transfer, as recommended by Bhatia et al. (1994).

Radioimmunoassay. Plasma samples were thawed on ice and two aliquots of 1.5 ml were mixed with 3 ml 98% ethanol (vol/vol), vortexed for 10 sec, and centrifuged at 2000 g for 15 min. The supernatant was poured into a 5 ml glass tube and dried overnight in a rotary evaporator. The dried extract of plasma for the CCK RIA was stored at -20°C, and the extract of plasma for the GLP-1 RIA was stored in a desiccator at 4°C.

Cholecystokinin RIA was performed as described in Chapter 2.2, 2.4, and 2.5 of this thesis.

The GLP-1 antibody (KMJ-01, Gift from Dr. McIntosh, Department of Physiology, University of British Columbia, Vancouver, Canada) was used at a final dilution of 1:80,000 (after addition of tracer) in a .1 M Tris assay buffer (pH 8.5) containing .05 M NaCl, .02 M EDTA .02% sodium azide (wt/vol), .2% BSA (wt/vol), and 67 KIU mg^{-1} Aprotinin (Trasylol, Bayer). The antibody is highly specific for the C-terminus of the GLP-1 (7-36) amide and does not bind to biologically inactive forms of GLP-1 which are secreted by the pancreas. It does however bind to the GLP-1 (9-36) amide, which is biologically inactive. Synthetic GLP-1 (7-36) amide (Peninsula Laboratories, Belmont, CA, USA) was iodinated with ^{125}I as described previously (Xia, 1996). Briefly, 5 to 10 μg of synthetic human GLP-1 (7-36) amide was dissolved in 10 μl distilled water and 10 μl of .5 M phosphate buffer (pH 7.5) added. Five microliters of

carrier-free ^{125}I-sodium iodide (Du Pont NEN Canada Inc., Mississauga, ON, Canada) and 10 μl chloramine-T (2 mg ml^{-1} in .04 M phosphate buffer, pH 7.5) were added to the reaction tube and mixed gently for 30 sec. The reaction was stopped by the addition of 10 μl of sodium metabisulphite (5 mg ml^{-1} in .04 M phosphate buffer, pH 7.5). A Sep Pak C-18 cartridge (Waters, Milford, MA, USA) was used to separate the ^{125}I-GLP-1 (7-36) amide. Iodinated GLP-1 amide was applied to the primed cartridge and washed with 10 to 60% acetonitrile (5 ml of 10% and 20%, 1 ml of 30%, 40%, and 50%, and 5 ml of 60%; vol/vol, respectively) containing .1 % trifluoroacetic acid (vol/vol). Fractions (10 μl) of the eluate were collected and counted in a gamma counter. The fractions containing the majority of the radioactivity (^{125}I-GLP-1 amide) were used in a binding test to confirm their ability to bind with the GLP-1 antibody KMJ-03. Iodinated GLP-1 (3000 cpm) was added to each assay tube after 24 h incubation of antibody and samples at 4°C. The total volume per assay tube was 800 μl. After incubation at 4°C for 48 h, free and bound tracer were separated by the addition of 100 μl assay buffer containing 8% (wt/vol) charcoal (Norit-A, Fisher), .4% (wt/vol) dextran (Sigma Chemical Company, St. Louis, MO, USA), .05 M EDTA, .05 M sodium barbitone (BDH Inc., Toronto, ON, Canada), and 2% BSA (wt/vol). Incubation time from addition of charcoal to separation by centrifugation (30 min, 10°C, 2000 g) was 10 to 15 min, limiting the number of tubes per assay to about 250. Subsamples (600 μl) of the supernatant were counted in a gamma counter. Inhibition curves were set up using the synthetic GLP-1 (7-36) amide (Peninsula Laboratories).

Data analysis and statistical evaluation. RNA abundance and blood concentration data were expressed as a percentage of baseline value for each animal prior to start of the experiment. The feed intake data measured over 24 h were expressed as 4, 8, 12, 16, 20, and 24 h cumulative intake curves. Data were analyzed using repeated measures analysis of variance with sources of variation of sampling time and error. The number of sampling times were 9, 10, and 6 for RNA abundance, blood concentration and cumulative feed intake, respectively. Computations were done with GLM procedure of SAS (1985).

4.3 Results

4.3.1 Effect of Fasting and Refeeding on CCK and Proglucagon Gene Expression

During the period of fasting the CCK mRNA abundance in the duodenum decreased continuously and then increased after refeeding so that the values following 8 h after refeeding were not different ($P<.05$) from the values prior to the start of the fasting period (FIGURE 4.1). The sample taken 12 h prior to fasting was used as a baseline value and all other values are expressed as a percentage of this first value. A photograph (FIGURE 4.2A) of the Northern transfer membrane showing Ethidium bromide-stained 28S rRNA reveals equal loading of similar amounts of RNA in different lanes. An autoradiogram of a complete Northern blot of duodenal biopsies (FIGURE 4.2B) from one animal shows the signal from CCK mRNA as well as non-specific signals of rRNA after 24 h exposure. The CCK mRNA signals for all five animals involved in the experiment are also shown (FIGURE 4.3) and it can be seen that the animal variation of the sample taken 12 h prior to fasting is very large. All animals, however, showed similar changes during fasting and refeeding periods if related to the pre-fasting value.

Proglucagon mRNA abundance in the ileum did not significantly change throughout the fasting and subsequent refeeding (FIGURE 4.4). FIGURE 4.5A depicts a complete autoradiogram of 15 µg total RNA isolated from the ileal biopsies from one animal. The autoradiogram shows the proglucagon mRNA signal and only very little non-specific interaction of the proglucagon riboprobe with rRNA. FIGURE 4.5B shows the proglucagon mRNA signals for the other animals with ileal cannulae. The sample taken 12 h prior to fasting is used as a baseline value and all other values are expressed as a percentage of this first value.

4.3.2 Effect of Fasting and Refeeding on CCK and GLP-1 Release

The concentration of pure peptide that produced half maximum inhibition of binding of tracer to antibody Deno was 2.7 pmol l^{-1} for CCK 8s, 29.8 pmol l^{-1} for porcine CCK 33s, and 1000 pmol l^{-1} for CCK 8ns. The intra- and interassay variability

coefficients were 4.85% and 5.57%, respectively. The detection limit of the assay defined as the smallest concentration of CCK in the assay tube which could be differentiated from absence of CCK with 95% confidence was .2 pmol l^{-1}. Recoveries with ^{125}I-labeled CCK were approximately 90%. The CCK blood concentrations of the analyzed samples ranged from 2.9 to 14.7 pmol l^{-1} which is in agreement with previously reported literature values (Furuse et al., 1991).

The concentration of pure peptide GLP-1 that produced half maximum inhibition of binding of tracer to antibody KMJ-03 was 200 pmol l^{-1}. The intra-assay variability coefficient was 5.4% (all samples of the experiment were run in the same assay; the inter-assay coefficient derived from three separate experiments was 33% due to difficulty of storage of GLP-1 containing samples). The detection limit of the assay was 18.94 pmol l^{-1}. Recoveries with ^{125}I-labeled GLP-1 were approximately 75%. The GLP-1 blood concentrations of the analyzed samples ranged from 68.9 to 306.7 pmol l^{-1}. We are not aware of GLP-1 blood concentrations previously reported in the mature bovine, Martin and Faulkner (1996) however reported lower GLP-1 concentrations in sheep (15 to 25 pmol l^{-1}).

Both the CCK and GLP-1 RIA results are expressed as relative change from the baseline value. Blood CCK concentration decreased markedly after the start of the fasting period and reached its lowest value at 24 h fasting. It then started to increase, even before the end of fasting, and continued to increase during the refeeding period. The CCK blood concentration from the start of the fasting period to 24 h into refeeding were all significantly lower than that in the original blood sample (FIGURE 4.6). The CCK concentration in blood taken at 36 h refeeding was not significantly different from the original blood CCK concentration.

The blood GLP-1 concentration also decreased with the beginning of fasting. Nadir values were observed at 12 to 24 h fasting and then the concentration returned to pre-fasting values after 8 h of refeeding (FIGURE 4.7).

4.3.3 Effect of 48 h Fasting on Rumen Content and Subsequent Feed Intake

Examination of the rumen content of the heifers through the ruminal cannulae after 48 h of fasting revealed that they contained a negligible amount of digesta confirming that this time period was sufficient for digestion and passage of digesta from the rumen to be near completion. The cumulative intakes during the first 16 h of the day of refeeding and on the following day did not differ from the intake during the same time on days before fasting (FIGURE 4.8). Within 24 h of refeeding, the animals consumed 75% of the feed they consumed before fasting, with the 20 and 24 h intake being significantly lower than on pre-fasting days ($P<.05$). At the end of the second day of refeeding intake had recovered to its pre-fasting amounts.

4.4 Discussion

The main observations in the present study are that fasting and refeeding induce changes in the abundance of CCK mRNA as well as the blood concentration of both CCK and GLP-1. These observations may reflect a role for CCK and GLP-1 in feed intake regulation in the bovine. Cholecystokinin mRNA abundance, CCK blood concentration, and GLP-1 blood concentration decreased during the period of fasting. However, by 8 to 36 h after refeeding all values had increased to the point where they were not statistically different from pre-fasting values. This data is in agreement with reported changes in CCK mRNA abundance and CCK blood concentration changes in rats during a 24 h fasting and refeeding period (Kanayama and Liddle 1991). Moreover, Hoyt et al. (1996) reported proglucagon mRNA abundance in the jejunum and ileum and GLP-1 blood concentration to decrease during a 72 h fasting period in rats. However, only the jejunum proglucagon mRNA abundance and the GLP-1 blood concentration returned to the pre-fasting values after 24 h refeeding, whereas the ileal proglucagon mRNA abundance remained at fasting values (Hoyt et al., 1996).

It is obvious that the CCK mRNA abundance relative to the time of feeding changes very rapidly. The heifer 5, shown at the bottom of figure 3 has the lowest signal

for CCK mRNA after 24 h fasting, unlike the other four animals having their lowest signal for CCK mRNA after 48 h fasting. This is due to the fact that the animal broke her chain after approximately 44 h fasting, left her tie stall and fed on some grain of other cows in the research facility. The 48 h fasting signal for CCK-mRNA of this heifer therefore represents more realistically a 4 h refeeding signal, and was very comparable to the other four heifers 4 h refeeding signal. During the analysis of the data we treated the mRNA and blood values of this heifer as if its 48 h fasting, 4, 8, 16, and 24 h refeeding values were 4, 8, 16, 24, and 36 h refeeding values.

The fasting- and refeeding-induced changes in abundance of CCK mRNA were paralleled by similar changes in CCK blood concentration. The finding that the CCK blood concentrations increased even before refeeding may be explained with a decrease of CCK-inactivating peptidases in the gastrointestinal tract and blood plasma in response to prolonged fasting.

In contrast to the CCK data, there were no detectable changes in the abundance of proglucagon mRNA even though fasting-and refeeding-induced changes in the blood concentration of GLP-1 similar to those seen for CCK. One interpretation of these observations is that there are substantial differences between the molecular mechanisms underlying the regulation of CCK and GLP-1. However, for a gastrointestinal hormone to fulfill any function related to regulating the digestion of food or controlling hunger or satiety its gene would have to be transcribed, its mRNA translated and the protein synthesized, modified, and released. Presumably nutrient interaction with gastrointestinal cells would influence one or some combination of these regulatory processes. Peripheral CCK and GLP-1 are known to be involved in the regulation of the pancreatic secretion, gastric acid secretion, and gastric emptying through vagal (Soudah et al., 1992; Wettergren et al., 1994) or paracrine mechanisms (Zabielski et al., 1994; Schmidt et al., 1994). A central role in regulating feed intake has also been proposed for CCK in several species (Anika et al., 1981; Della Fera and Baile, 1980; Gibbs et al., 1973; Gregory et al., 1989; Kissileff et al., 1981; and Lieverse et al., 1994) and for GLP-1 in rats (Turton et al., 1996). The fasting-and feeding-induced changes observed for CCK and GLP-1 in the

present study indicate that both these gastrointestinal hormones have a role in regulating food digestion and possibly controlling feed intake in ruminants.

Greenstein et al. (1990) showed that in rats after 5 days of fasting the CCK mRNA abundance in the duodenum decreased in a manner that paralleled weight reduction of the duodenum whereas CCK mRNA abundance remained constant if expressed relative to a unit of total RNA. Our biopsy sampling technique did not allow us to determine whether there was a weight change in the duodenum or ileum of the heifers after two days of food deprivation. The size of the intestinal biopsies appeared to remain consistent, though we did not weigh the individual biopsies. There was no change in the yield of total RNA from intestinal tissue taken during fasting or refeeding. As a result, the analysis of an equal amount of total RNA from each collection time revealed a reduction in CCK mRNA abundance during the fasting period, which was paralleled by a reduction in CCK release. These results indicate that there is a relationship between the abundance of CCK mRNA and the synthesis and release of the CCK protein. Since this relationship was revealed by fasting and refeeding, it seems reasonable to suggest that nutrients in the gastrointestinal tract were at least partly responsible for the observed changes. It also follows that there is a molecular detection system that not only responds to nutrients but also signals or interacts with gastrointestinal hormone genes such as the CCK gene.

For proglucagon, the relationship between mRNA abundance and GLP-1 release appears to be different than for CCK. Very little is known about storage, transport, and release of CCK or GLP-1 in mucosa cells. One explanation could be that GLP-1 is stored in cytosolic vesicles. This would allow for an immediate GLP-1 response to nutrients reaching the intestine without the need for proglucagon gene transcription. Data presented by Ørskov and Holst (1987) indicating that different amounts of GLP-1 and GLP-2 were released in response to a mixed meal in humans in spite of equimolar amounts of both peptides being produced from the preproglucagon peptide in the intestine would support the hypothesis of storage of GLP-1 peptides in the cytosol.

The small amount of digesta remaining in the rumen after 48 h of fasting indicates that this length of time was sufficient for digestion and passage of food to the intestine to

be negligible. The feed intake data reveal that the cumulative intake during 2 to 16 h refeeding after 48 h fasting was not significantly different from the intake before fasting despite the fact that CCK and GLP-1 blood concentrations remained low for 24 and 4 h respectively. The feed intake after 20 and 24 h during the first day following fasting was significantly reduced ($P < .05$). The feed intake during the second day following fasting was not different from the pre-fasting intake. These findings conflict with the expectation that reduced release of an intestinal short-term satiety factor such as CCK would lead to increased short-term feed intake. In rats an increased feed intake due to increased meal size for a period of 10 days was reported following a 72 h fasting period (Del Prete et al., 1994). However, it is possible that the heifers compensated for the reduced energy intake to some extent by a reduced rate of metabolism, which would allow them to make up for the reduced intake over a longer period of time without an increased short term feed intake. It is also likely that other limitations following 48 h of fasting caused a reduced 24 h feed intake on the first day after refeeding. The limitations may be related to a reduced rumen microbial population at the start of the refeeding period and (or) to the lack of normal rumen distention during fasting. Consistent with these possibilities is the likelihood that digesta reaching the small intestine is not the only mechanism responsible for feed intake regulation in the ruminants. However, this does not preclude a role for intestinal satiety factors in ruminants during a normal meal situation, in particular with feeds containing a high amount of rumen-bypass nutrients, which reach the intestine without changing rumen fermentation.

Further research is required to characterize the influence of different nutrients on the regulatory processes that influence gastrointestinal hormone genes and the fate of the proteins they encode. Such information will contribute to an understanding of which nutrients and hormones have the most important roles in controlling feed intake in ruminants.

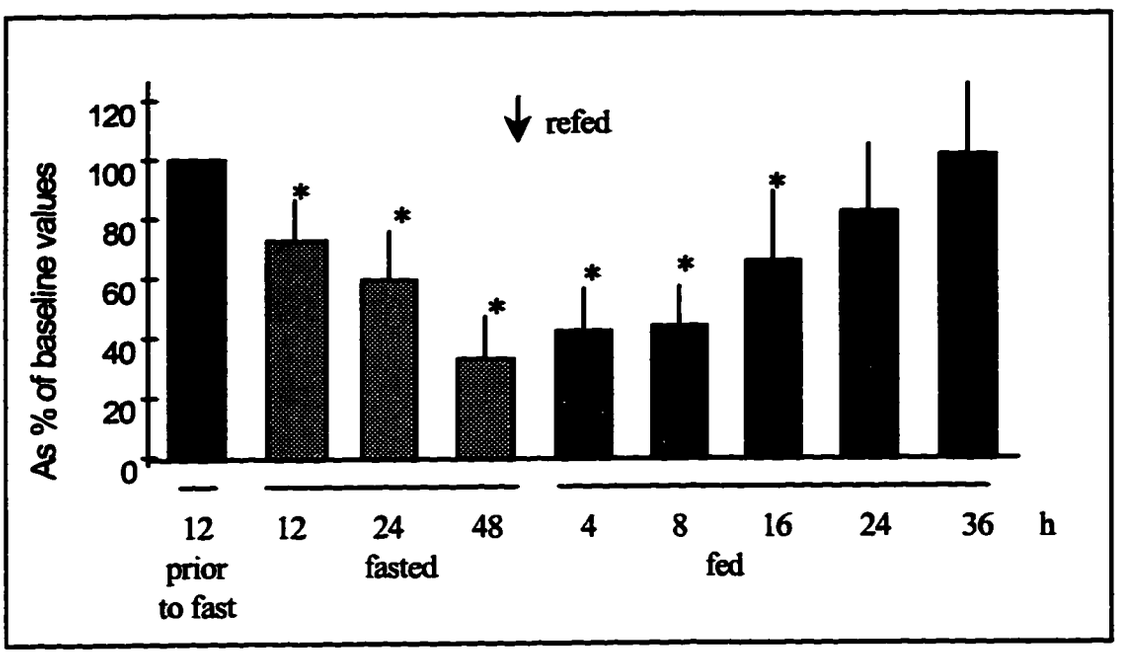

FIGURE 4.1 - Effect of Fasting and Refeeding on CCK mRNA Abundance in Bovine Duodenal Mucosa (Graph)

Effect of 48 h fasting and subsequent refeeding on CCK mRNA abundance in the bovine duodenum. Each sampling point is expressed as a percentage of the baseline value, which was derived from samples obtained 12 h before the start of fasting to compensate for variability among animals in basal CCK mRNA abundance. The CCK mRNA abundance in the biopsies taken 12 h prior to fasting ranged from .74 to 1.42 OD units. (Bars: means; error bars: SD; $n = 5$; * $p < .05$).

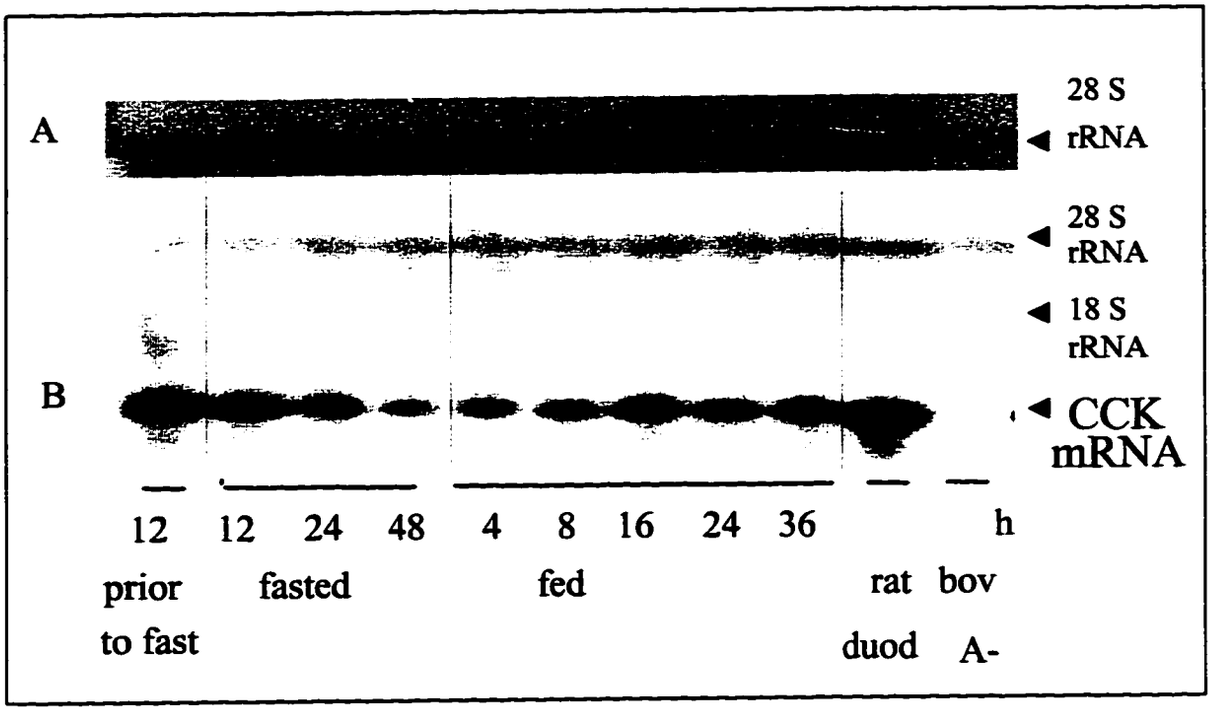

FIGURE 4.2 - Complete Autoradiogram of Northern Hybridization of RNA Isolated from Duodenal Mucosa Biopsies with CCK Riboprobe

Autoradiogram depicting the effect of 48 h fasting and refeeding on CCK mRNA abundance in duodenal mucosa (B). Twenty micrograms of total RNA derived from duodenal biopsies taken at each sampling time from one heifer, 20 μg rat duodenum RNA (positive control), and 20 μg bovine duodenum RNA depleted of polyadenylated (A^-) RNA (negative control) are shown. The location of 28S and 18S rRNA and CCK mRNA are indicated. Autoradiographic exposure was 19 h. A photograph of the Northern transfer membrane is presented (A). Ethidium bromide-stained 28 S rRNA reveals equal loading of similar amounts of RNA in different lanes.

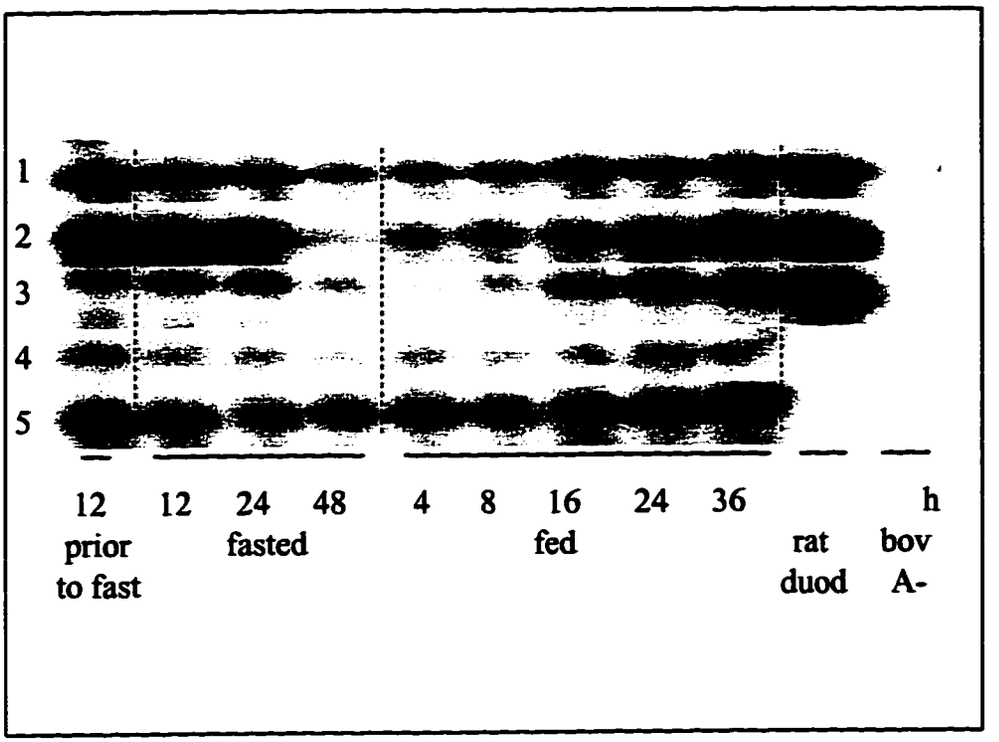

FIGURE 4.3 - Effect of Fasting and Refeeding on CCK mRNA Abundance in Bovine Duodenal Mucosa (Autoradiogram)

Autoradiogram depicting CCK mRNA abundance in duodenal mucosa of 5 heifers in response to fasting and refeeding. Twenty micrograms of total RNA derived from duodenal biopsies taken at each sampling time from each heifer, 20 μg rat duodenum RNA (positive control), and 20 μg bovine duodenum RNA depleted of polyadenylated (A^-) RNA (negative control) are shown. Autoradiographic exposure was 19 h.

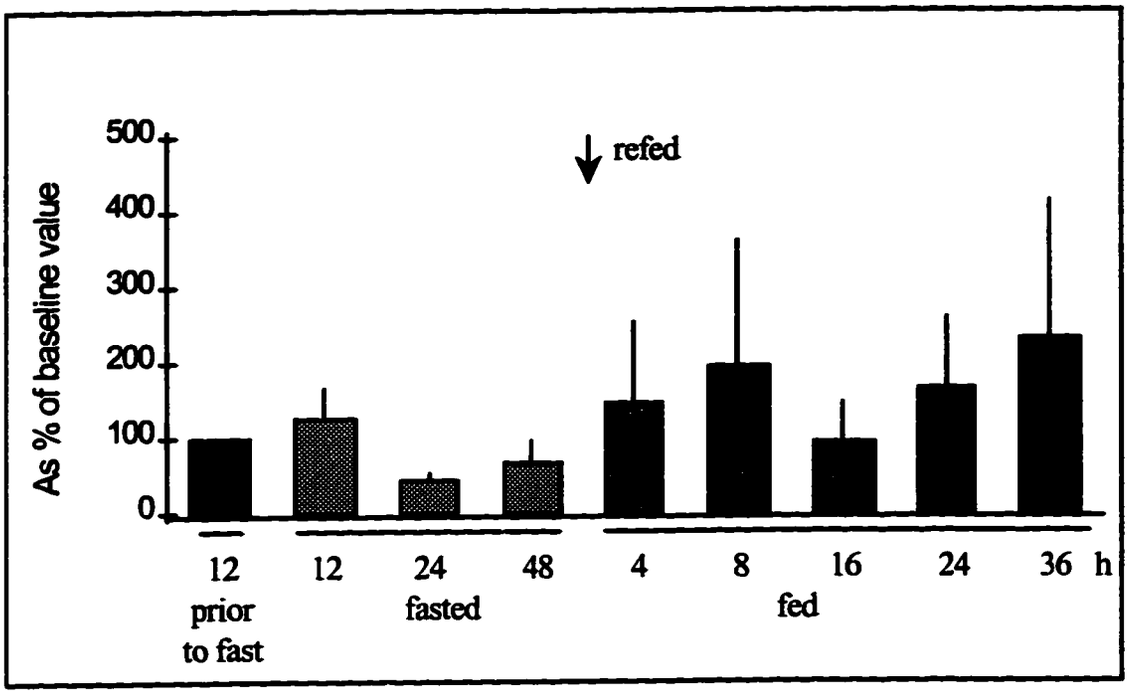

FIGURE 4.4 - Effect of Fasting and Refeeding on Proglucagon mRNA Abundance in Bovine Ileal Mucosa (Graph)

Effect of 48 h fasting and refeeding on proglucagon mRNA abundance in the bovine ileum. Each sampling point was expressed as a percentage of the baseline value which was derived from samples obtained 12 h before the start of fasting to compensate for variability among animals in basal CCK mRNA abundance. The proglucagon mRNA abundance in the biopsies taken 12 h prior to fasting ranged from 0.94 to 3.12 OD units. (Bars: means; error bars: SD; n = 3).

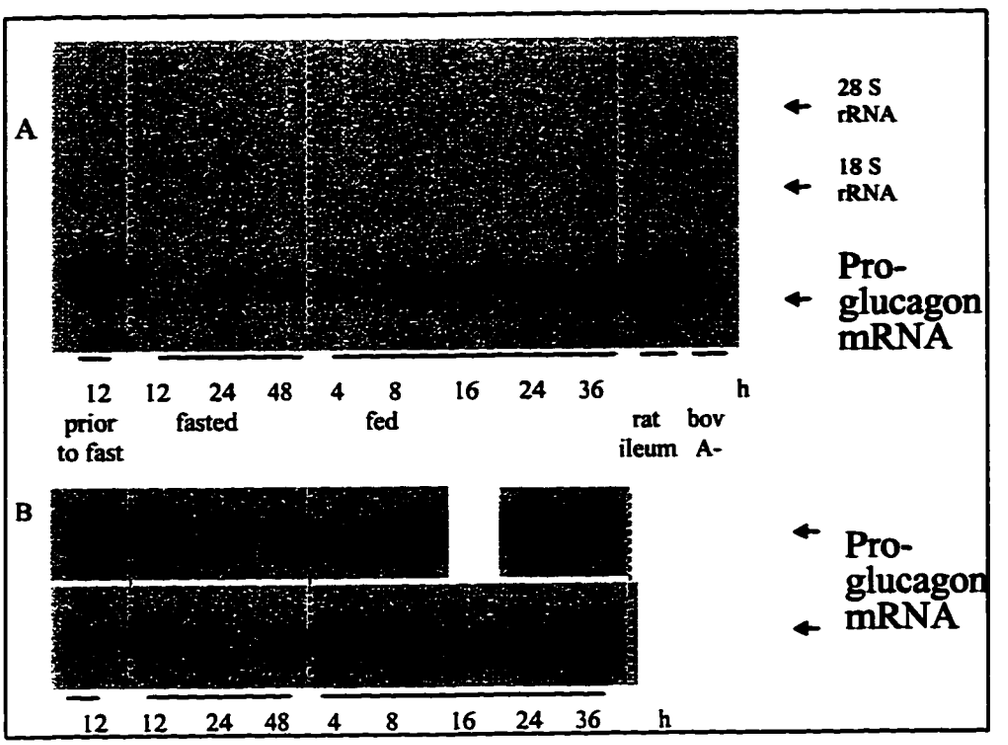

FIGURE 4.5 - Effect of Fasting and Refeeding on Proglucagon mRNA Abundance in Bovine Ileal Mucosa (Autoradiogram)

Autoradiogram depicting the effect of 48 h fasting and refeeding on proglucagon mRNA abundance in ileal mucosa. Fifteen micrograms of total RNA derived from ileal biopsies taken at each sampling time from one heifer, 15 μg rat ileum RNA (rat ileal; positive control), and 15 μg bovine ileum RNA depleted of polyadenylated RNA (bov A^-; negative control) are shown (A). The location of 28S and 18S rRNA and proglucagon mRNA are indicated. Autoradiographic exposure was 5 h. Autoradiograms showing proglucagon mRNA abundance of the other two heifers are also presented (B).

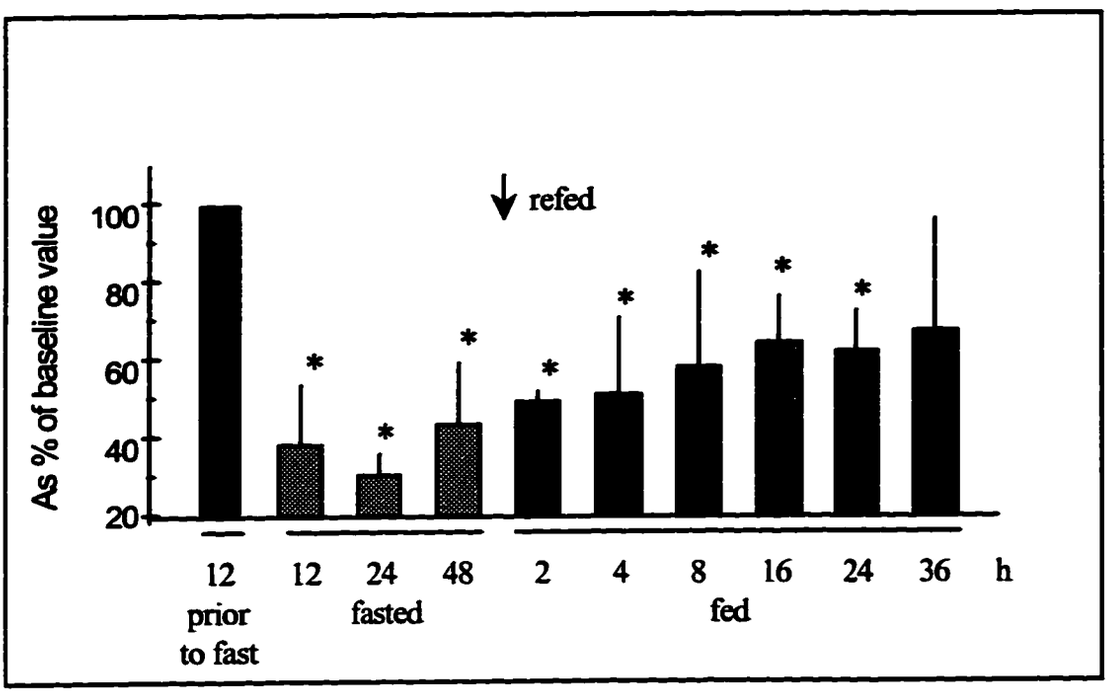

FIGURE 4.6 - Effect of Fasting and Refeeding on Bovine CCK Blood Concentration

Effect of 48 h fasting and refeeding on CCK blood concentration. Each sampling point was expressed as a percentage of the baseline value which was derived from samples obtained 12 h before the start of fasting to compensate for variability among animals in basal CCK concentration. The CCK blood concentrations in the samples taken 12 h prior to fasting ranged from 11.3 to 14.7 pmol l^{-1}. (Bars: means; error bars: SD; n = 5; * $p < .05$).

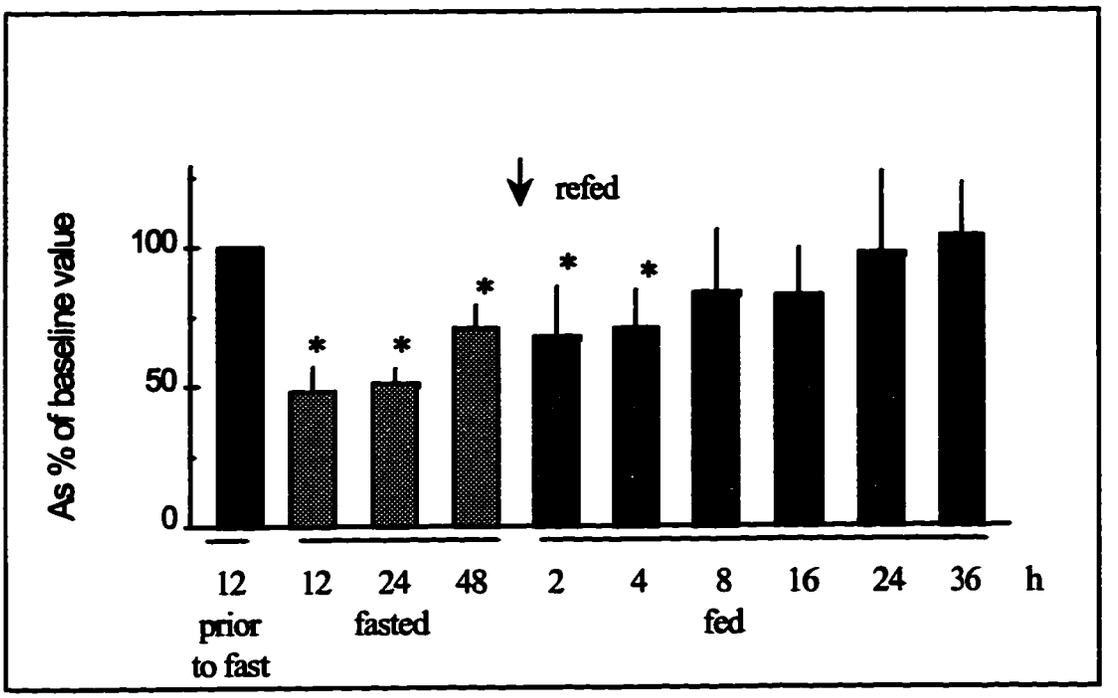

FIGURE 4.7 - Effect of Fasting and Refeeding on Bovine GLP-1 Blood Concentration
Effect of 48 h fasting and refeeding on GLP-1 blood concentration. Each sampling point was expressed as a percentage of the baseline value which was derived from samples obtained 12 h before the start of fasting to compensate for variability among animals in basal GLP-1 concentration. The GLP-1 blood concentrations in the samples taken 12 h prior to fasting ranged from 179.5 to 240.2 pmol l^{-1}.(Bars: means; error bars: SD; n = 5; * $p < .05$).

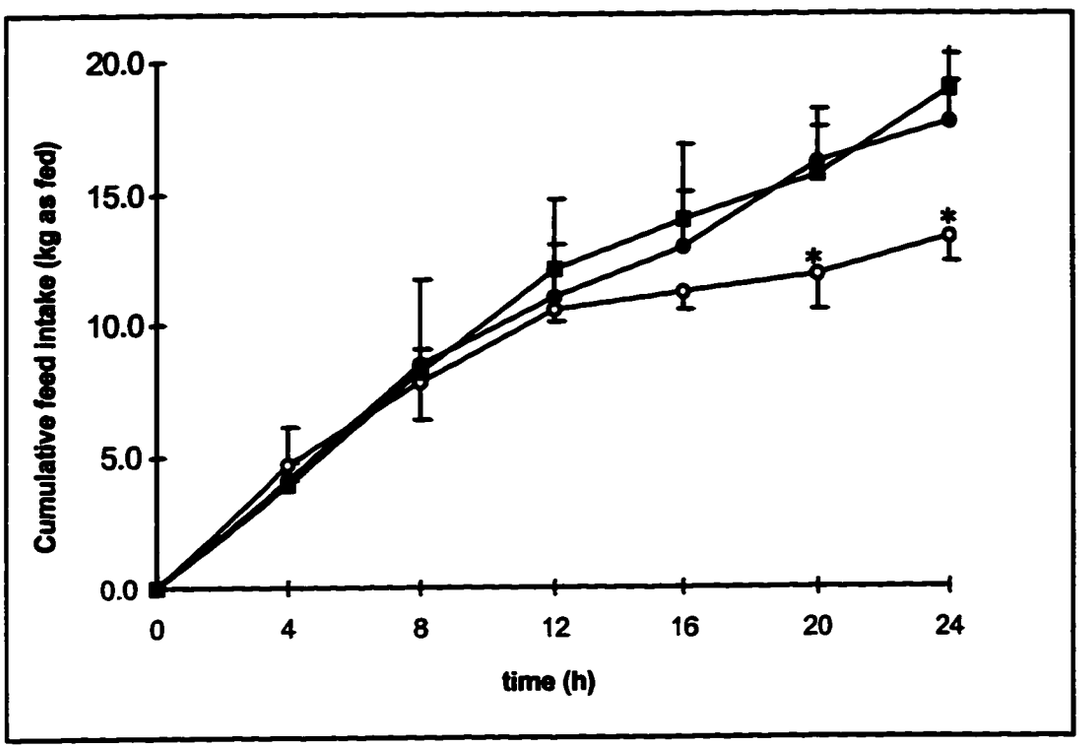

FIGURE 4.8 - Effect of Fasting and Refeeding on Feed Intake in Dairy Heifers

Effect of 48 h fasting and refeeding on cumulative feed intake of dairy heifers measured by automatic feed scales. Average cumulative intake three days before fasting (●), the day of refeeding ( ), and the following day (■) are shown (Data points: means; n = 4; error bars: SD; * $p < .05$).

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Chapter 5 - Intestinal Nutrient-Gene Interaction: Effect of Abomasal Infusions of Different Nutrients and Soybean Trypsin Inhibitor and Duodenal Infusion of Trypsin on Cholecystokinin and Proglucagon Gene Expression

5.1 Introduction

Cholecystokinin (CCK) is a neuropeptide found primarily in I-cells in the proximal small intestine (Buffa et al. 1976) and in the brain (Dockray, 1976). It is released in response to food ingestion in for example, humans, pigs, rats, and other species as reviewed by Rehfeld (1986). Functions of CCK of intestinal origin include gallbladder contraction (Ivy and Oldberg, 1928) and pancreatic digestive enzyme secretion (Reidelberger et al., 1986; Rosewicz et al., 1989), but it has also been associated with eating disorders (Geracioti and Liddle, 1988). A role for CCK as a satiety factor has been studied in several species including rats (Gibbs et al., 1973), pigs (Anika et al., 1981; Ebenezer et al., 1989), sheep (Della-Fera and Baile, 1980, Farningham 1991), and humans (Kissileff, et al., 1981; Lieverse, et al., 1994).

In different species specific nutrients and nutritional factors have been shown to induce or inhibit CCK release. Dietary regulation of intestinal CCK gene expression and CCK release has been demonstrated with soybean trypsin inhibitor in rats (Liddle et al., 1988, Rosewicz et al., 1989). In addition, duodenal infusion of lipids and diets high in fat induced CCK-release, which reduced short term feed intake in humans (Owyang et al., 1986), pigs (Gregory et al., 1988), and in lactating cows (Choi and Palmquist, 1996). Duodenal infusions of phenylalanine or diets high in phenylalanine increase plasma CCK concentrations in man (Owyang et al., 1986); goats, and chickens (Furuse et al., 1991a, and 1991b), and increased dietary protein stimulates CCK secretion in rats (Liddle et al., 1986). Owyang et al. (1986) also showed that intraduodenal trypsin infusion can reduce meal-induced CCK release in humans. Glucose and sucrose administration enhances

CCK release in humans (Hasegawa et al., 1994) and synergism exists between propionate and CCK in the control of feed intake in sheep (Farningham et al., 1993).

Glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) are the major intestinal proglucagon product secreted from the L-cells predominantly in the distal ileum of monogastric species (Ørskov et al., 1986). Glucagon-like peptide-2 was shown to affect ileal hexose transport in rats (Cheeseman and Tsang, 1996) and several functions have been demonstrated for GLP-1. Glucagon-like peptide-1 is insulinotropic (Holst et al., 1987), glucagonostatic (Komatsu et al., 1989), and inhibits gastric acid secretion (Wettergren et al., 1994) as well as gastric emptying (Wettergren et al., 1993). A role for GLP-1 in the central regulation of feeding has also been suggested even though peripherally administered GLP-1 did not affect feed intake (Turton et al., 1996). Hoyt et al. (1996) demonstrated that in rats proglucagon mRNA abundance and GLP-1 release decrease during a 24 h fasting period and return to pre-fasting values 24 h after refeeding. In sheep, GLP-1 has been shown to induce an increase in blood insulin concentration during hyperglycaemic conditions comparable to that induced by postprandial blood glucose (Martin and Faulkner, 1993).

Traditionally feed intake regulation in ruminants is believed to occur primarily at the level of the first stomach, the rumen. Rumen fill, passage rate of digesta through the forestomachs, and accumulation of ruminal fermentation products are key components of feed intake control in ruminants (Baile et al., 1988). However, evidence is accumulating that there are other factors exerting control over the outflow of digesta from the reticulorumen and that control of digesta outflow occurs after the reticulo-omasal orifice (Malbert and Ruckebusch, 1989; Mathison et al. 1995). Although small intestinal feedback mechanisms regulating feed intake have been actively researched in monogastrics, very little work has been undertaken in ruminants. As a result, information regarding factors regulating CCK and GLP-1 in ruminants are limited. However, as reported in Chapter 4 of this thesis we demonstrated that CCK and GLP-1 are released in response to refeeding after fasting which satisfies one of the five criteria established by Smith (1984) for a physiological satiety effects of a gut hormone. Our previous data

provide support for the hypothesis that feedback from nutrients delivered to the small intestine plays a role in feed intake regulation in ruminants. In the present study we investigated the effect of supplementary infusion of nutrients in fed animals on the regulation of CCK and proglucagon gene expression during two 4 x 4 Latin square experiments consisting of abomasal infusion of nutrients or soybean trypsin inhibitor, or duodenal infusion of trypsin.

5.2 Materials and Methods

5.2.1 *Animals and Sampling Procedures*

The experiment was carried out using four Holstein heifers (550 ± 50 kg BW) surgically fitted with ruminal, duodenal, and ileal cannulae, abomasal infusion lines and jugular catheters as described in Chapter 3.2 and 4.2 of this thesis. The animals were housed in tie stalls with access to water at all times and were accustomed to once a day feeding (0900 h) of a total mixed ration (TMR) balanced according to NRC (1989) requirements. The amount of feed consumed was continuously recorded with automatic feed scales.

A fiberoptic endoscope and biopsy forceps were used to obtain duodenal and ileal tissue samples and blood samples, all of which were collected and stored as described in Chapter 3.2 and 4.2 of this thesis.

5.2.2 *Experimental Design*

Two 4 x 4 Latin square design experiments were completed. Treatments were infused once a week for four hours and consisted in the first Latin square of abomasal infusion of saline (65 g h^{-1}), canola oil (20 g h^{-1}), phenylalanine (.15 M; 150 g h^{-1}), and soybean trypsin inhibitor (SBTI, 150 mg l^{-1}; 65 g h^{-1}). For the second Latin square experiment the treatment consisted of abomasal infusion of saline (750 g h^{-1}), casein (100 g l^{-1}; 750 g h^{-1}), starch (partially hydrolyzed; 50 g l^{-1}; 900 g h^{-1}), and duodenal infusion of trypsin (1.5 g l^{-1}; 75 g h^{-1}). In each period, feed was withheld from the heifers four hours

prior to the start of the infusions and feed was offered with the start of the infusion. Three duodenal biopsies were taken approximately 3 to 5 cm apart 2 h prior to the infusion (baseline value), and then 1, 2, 4, and 24 h after start of the infusion. Two ileal biopsies were taken 3 to 5 cm apart 2 h prior to the infusion (baseline value) and then 3, 5, and 25 h after the start of the infusion. Blood samples were collected 1 and .5 h prior to the infusion (baseline), and then .5, 1, 1.5, 2, 4, 8, 12, 16, 20, and 24 h after the start of the infusion (12 and 20 h only for the first Latin square).

5.2.3 *Assays*

5.2.3.1 *Isolation of RNA, gel-electrophoresis, and Northern transfer.*

Total RNA was isolated from the intestinal biopsies with the TRIzol® method (Gibco Life Technologies, Burlington, ON, Canada). Two ileal or three duodenal biopsies collected at each sampling time from each animal were pooled, homogenized with TRIzol reagent and the RNA isolation completed as described in Chapter 3.2 of this thesis. The RNA was resuspended in TE buffer and quantified by UV absorption at 260 nm. The UV absorbance ratio (260/280 nm) of RNA ranged from 1.89 - 2.03.

Total RNA was isolated from intestinal tissue using a standard GIT-CsCl procedure as described previously (Glimm et al., 1990). Polyadenylated RNA (poly A^+ RNA) was isolated from duodenal total RNA as described previously (Glimm et al., 1990) using LiCl buffer and oligo(dT)-cellulose chromatography. Twenty micrograms of RNA depleted of polyadenylated RNA (poly A^- RNA) was used as a negative control. Twenty micrograms of total RNA isolated from rat duodenal mucosa or 15 μg total RNA isolated from rat ileal mucosa were used as positive controls in each of the Northern hybridizations probed for CCK and proglucagon respectively to allow comparison between membranes.

Twenty micrograms of total RNA from the duodenal biopsy samples or 15 μg of total RNA from the ileal biopsy samples of each animal and the positive and negative control were separated by electrophoresis in denaturing agarose gels and the RNA

transferred to Zeta-probe nylon membranes (Life Science, Bio-Rad, Mississauga, ON, Canada) as described previously (Glimm et al., 1990). The membranes were baked under vacuum at 80 °C for 2 h.

5.2.3.2 *Radiolabeled riboprobes and Northern hybridization.*

The CCK and the proglucagon antisense riboprobes were generated as described in Chapter 3.2 and 4.2 of this thesis and the riboprobe purified by phenol-chloroform extraction and Sephadex G-50 (DNA grade fine, Pharmacia, Baie D'Urfe, QE, Canada) chromatography as described previously (Glimm et al., 1990). Membranes were prehybridized and then those with duodenal RNA hybridized using the CCK riboprobe and those with ileal RNA were hybridized using the proglucagon riboprobe. Both riboprobes were used at 2 x10^6 dpm/ml of hybridization solution. After hybridization the membranes were washed as described and autoradiography of Northern blots was performed as described by Glimm et al. (1992) and in Chapter 3.2 and 4.2 of this thesis. The autoradiograms were analyzed by imaging densitometry and the values for each band were corrected for loading differences based on the intensity of the corresponding Ethidium bromide stained 28S rRNA band on the membrane after completing the transfer. The rat total RNA sample which was loaded on each gel as an internal standard was used to calculate the interassay variation coefficient from the standard deviation of the internal standard sample between each membrane divided by the mean corrected volume.

5.2.3.3 *Radioimmunoassay.*

Plasma samples were thawed on ice and two aliquots of 1.5 ml were mixed with 3 ml 98% ethanol (vol/vol), vortexed for 10 sec, and centrifuged at 2000 *g* for 15 min. The supernatant was poured into 5 ml glass tubes and dried overnight in a rotary evaporator. The dried extract of plasma for the CCK RIA was stored at -20°C, and the extract of plasma for the GLP-1 RIA was stored in a desiccator at 4°C.

Cholecystokinin RIA was performed as described in Chapter 2.2, 2.4, and 2.5 of

this thesis and the GLP-1 RIA as described in Chapter 4.2 of this thesis.

5.2.4 *Data Analysis and Statistical Evaluation*

RNA abundance and blood concentration data were expressed as a percentage of baseline values obtained before the start of the experiment. The feed intake data two days prior to and two days after the infusion were expressed as 24 h cumulative feed intake. On the day of infusion data were expressed as 2, 4, 8, and 24 h cumulative feed intake. Data were analyzed using repeated measures analysis of variance. Whole plot sources of variation were animal, period, treatment, and residual whole plot variation (E_1), and sub-plot sources of variation were repeated measures of sampling time, sampling time * animal, sampling time * period, sampling time * treatment, and sub-plot error (E_2). Whole plot sources were tested against E_1 and sub-plot sources against E_2. The number of sampling times were 5, 11, 9, 4, 8, and 3 for CCK RNA abundance, CCK blood concentration for the first Latin square, CCK blood concentrations for the second Latin square, Proglucagon RNA abundance, GLP-1 blood concentration, and 24 h cumulative feed intake respectively. Contrasts between the first sampling time and each of the other sampling times were made within the repeated measures of time. The 2, 4, and 8 h cumulative feed intake data were each separately analyzed with the whole plot sources of variation animal, period, treatment and residual plot variation (E_1). Computations were done using the GLM procedure of SAS (1985).

5.3 Results and Discussion

5.3.1 *Effect of Altered Intestinal Digesta Composition on CCK and Proglucagon Gene Expression*

The interassay coefficient was 4% for the membranes hybridized with CCK riboprobe and 1.2% for the membranes hybridized with the proglucagon riboprobe. An autoradiogram of a complete Northern blot of duodenal biopsies from one animal shows the signal from CCK mRNA as well as non-specific signals of rRNA after 24 h exposure

(FIGURE 5.1A). A photograph (FIGURE 5.1B) of the Northern transfer membrane showing Ethidium bromide-stained 28S rRNA reveals equal loading of similar amounts of RNA in different lanes. An autoradiogram of a Northern blot of proglucagon mRNA from ileal biopsies shows very little non-specific interaction of the proglucagon riboprobe with rRNA (FIGURE 5.2A). A photograph of the Northern transfer membrane with Ethidium bromide-stained 28S rRNA is also shown and reveals equal loading of similar amounts of RNA in different lanes (FIGURE 5.2B).

The results of the two Latin square experiments on the effect of altered digesta composition on CCK and proglucagon mRNA abundance are presented in FIG. 5.3A, 5.3B, 5.4A and 5.4B. The CCK mRNA abundance was not different in animals infused with SBTI, canola oil, phenylalanine, or saline in the first Latin square (FIGURE 5.3A) or in animals infused with casein, starch, trypsin, or saline in the second Latin square (FIGURE 5.3B). The infusion amounts for each infusate were calculated to be equal or greater than that supplied by the diet so that substrate delivery to the duodenum was essentially doubled. In a preliminary study (Chapter 3) we used the same technique to infuse canola oil for four hours into the abomasum, to double or triple the amount of oil reaching the small intestine in three lactating dairy cows we measured a maximal response of CCK mRNA abundance after one to two hours. Furuse et al. (1991) measured differences in CCK blood concentration following phenylalanine infusion one hour after start of infusion, and Choi et al. (1996) also reported changes in CCK blood concentration three to four hours after feeding. In sheep, soybean trypsin inhibitor has also been suggested to increase CCK release (Kato et al., 1986) although they did not measure changes in CCK blood concentration. However, SBTI has been shown to cause a pancreatic response, which is one of the functions of CCK in monogastrics (Soudah et al., 1992) and young ruminants (Zabielski et al., 1994). Our findings from Chapter 4 indicated that both an increase in CCK release and CCK mRNA abundance could be seen as soon as 4 h after refeeding following a 48 h fast. We had expected that a four hour infusion period would alter the digesta composition sufficiently to cause a change in CCK mRNA abundance in fed animals. However, we could not confirm our hypothesis

that dietary components reaching the intestine cause a change in CCK mRNA abundance in the fed animals in the present study.

Due to problems with the ileal cannula of one animal, only three of the four heifers could be used to collect ileal biopsy samples and to measure proglucagon mRNA abundance. Ileal proglucagon mRNA abundance did not differ in animals infused with SBTI, canola oil, phenylalanine, or saline in the first Latin square (FIGURE 5.4A). In addition, animals infused with casein, starch, trypsin, or saline in the second Latin square did not show differences in proglucagon mRNA (FIGURE 5.4B). Information on the influence of nutrients on GLP-1 release or proglucagon mRNA abundance is limited (Tappenden et al., 1996). In rats the ingestion of dietary fiber for two weeks is associated with increases in intestinal proglucagon mRNA abundance (Reimer and McBurney, 1996; Reimer et al., in press). Hoyt et al. (1996) reported that proglucagon mRNA abundance decreased during 24 h fasting in the rat jejunum and ileum and increased to pre-fasting values within 24 h of refeeding in the jejunum, but not in the ileum. In the same study Hoyt et al. (1996) reported that intra-jejunal infusion of triglycerides increased the expression of proglucagon. Ileal proglucagon gene expression and plasma GLP-2 concentration increased within 6 h of addition of volatile fatty acids to total parenteral nutrition formulations infused into rats. (Tappenden et al., 1997). In humans, fats and glucose have been shown to induce a GLP-1 release (Roberg and Brubaker, 1991; Elliot et al., 1993). Our finding from Chapter 4 indicate that the ileal proglucagon mRNA abundance in growing Holstein cattle does not change during fasting and refeeding.

There are a number of possible explanations for the results observed in this study. Animal variation was larger than expected and this may have been a contributing factor in the lack of significant difference in response to the treatments. However, the experimental design helped to correct for animal variation and the baseline mRNA abundance values for each animal before the start of infusion was used to correct treatment values. The earliest response in proglucagon mRNA abundance to nutrients is 6 h (Tappenden et al., 1997), thus it may be that the duration of infusion and (or) delivery

of nutrients to the ileum was insufficient to increase proglucagon gene expression in values.

The lack of change in CCK or proglucagon mRNA abundance indicates either a lack of change of rate of transcription or altered transcription paralleled by altered turnover of mRNA. However, the above explanation is not sufficient to exclude a change of CCK or proglucagon response to the specific nutrient supplementation. The possibilities of modified translation, post-translational processing, protein turnover, receptor binding, or number of receptors in response to nutrient fluxes would have to be eliminated, before a lack of treatment effect can be verified. Liddle et al. (1988) demonstrated that in rats an increased plasma CCK concentration in response to duodenal trypsin inhibitor infusion was followed not only by increased CCK mRNA abundance but was also accompanied by an increase in CCK gene transcription. They reported that CCK plasma concentration reached the maximum level as soon as 4 h after the start of infusion, whereas CCK mRNA abundance increased by 200% after 4 h, 350% after 12h, and 400% after 24 h. In addition the transcriptional activity relative to beta-actin was highest after 4h, but maintained elevated levels throughout all of the study (Liddle et al., 1988). Shirazi-Beechey (1995) indicated that the time needed for complete cell renewal of enterocytes in the intestinal lumen varies between species from 2 to 3 days in rodents, 3 to 4 days in ovine, to 5 to 6 days in humans: no values for bovine were given. These species differences may explain the differential response in gastrointestinal gene expression to nutrient fluxes in ruminants relative to rodents. Prolonged infusions of specific nutrients into the abomasum of cattle may therefore be necessary to induce the changes in gastrointestinal hormone mRNA abundance observed with short-term nutrient infusion in monogastrics.

Kanayama and Liddle (1991a) showed that the stimulation of CCK secretion by neuro-hormonal mechanisms (intra-venous bombesin) in rats is not sufficient to increase CCK mRNA. However, they did not determine if there were changes in transcription rate. Several studies have reported a decrease in CCK mRNA abundance during food deprivation in rats (Kanayama and Liddle, 1991b; Koop et al., 1987; Greenstein et al.,

1990). Cattle are the only other species in which changes in CCK mRNA in response to fasting (Chapter 4 of this thesis). Friedman et al., (1985) showed differential expression of the CCK gene during brain and gut development in mice, but they did not investigate if transcription, translation, CCK-receptor abundance, or CCK-receptor binding changed. Although proglucagon mRNA abundance in rats was demonstrated to change in response to fasting and refeeding (Hoyt et al., 1996) fiber intake (Reimer and McBurney, 1996), and fiber fermentability (Reimer et al., in press), whether these factors also change the rate of proglucagon gene transcription has not yet been investigated.

Monitoring of blood peptide concentration can clarify if release of the peptide was triggered by nutrient supplementation, though questions about changes in post-transcriptional events, protein turnover, receptor abundance, and receptor binding cannot be answered by this approach.

5.3.2 *Effect of Altered Intestinal Digesta Composition on CCK and GLP-1 Release*

The concentration of pure peptide that produced half maximum inhibition of binding of tracer to antibody Deno was 2.7 pmol l^{-1} for CCK 8s, 29.8 pmol l^{-1} for porcine CCK-33s, and 1000 pmol l^{-1} for CCK-8ns. The intra- and interassay variability coefficients were 7.77% and 8.26%, respectively. The detection limit of the assay defined as the smallest concentration of CCK in the assay tube which could be differentiated from absence of CCK with 95% confidence was .2 pmol l^{-1}. Recoveries with ^{125}I-labeled CCK were approximately 90 %. The CCK blood concentration of the analyzed samples ranged from 2.29 to 38.1 pmol l^{-1}, which is in agreement with values previously reported in the literature (Choi et al., 1996).

The concentration of pure peptide GLP-1 that produced half maximum inhibition of binding of tracer to antibody KMJ-03 was 200 pmol l^{-1}. The intra- and interassay variability coefficients were 10.0% and 12.45% (the samples of each experiment were run in the two assays, each animal treatment being represented equally in each assay; the interassay coefficient derived from the two separate experiments was 31.7% , possibly due to degradation of GLP-1 in stored samples). The detection limit of the assay was 18.9

pmol l^{-1}. Recoveries with ^{125}I-labeled GLP-1 were approximately 75%. The GLP-1 blood concentration of the analyzed samples ranged from 28.2 to 239.3 pmol l^{-1} and are in agreement with the GLP-1 blood concentrations reported in Chapter 4 of this thesis. The binding of the GLP-1 antibody to the biologically inactive GLP-1 (9-36) peptide may cause these values to be artificially high. Martin and Faulkner (1996) reported lower values (25 to 15 pmol l^{-1}) in fed sheep.

The CCK and GLP-1 RIA results are expressed as relative change from the baseline value. The results of the two Latin square experiments on the effect of altered digesta composition on the CCK and GLP-1 blood concentration are presented in FIG. 5.5A, 5.5B, 5.6A and 5.6B, respectively.

The CCK blood concentration was not different between animals infused with SBTI, oil, phenylalanine, or saline in the first Latin square (FIGURE 5.5A) or for the animals infused with casein, starch, trypsin, or saline in the second Latin square (FIGURE 5.5B). These findings appear to contradict previous reports of phenylalanine, fat, or soybean trypsin inhibitor stimulating CCK secretion in ruminants (Furuse et al., 1991, Choi et al., 1996 and Kato et al., 1986). These results also contradict our findings that fasting and refeeding affects CCK secretion in dairy heifers (Chapter 4).

The GLP-1 blood concentration was also not different between animals infused with SBTI, oil, phenylalanine, or saline in the first Latin square (FIGURE 5.6A) or for the animals infused with casein, starch, trypsin, or saline in the second Latin square (FIGURE 5.6B). The phenylalanine infusion indicated a trend for increased GLP-1 release after 1 h 30 min ($p=0.069$) and 2h ($p=0.091$), but no treatment induced significant changes in GLP-1 release.

In monogastrics, the most important stimulus of GLP-1 secretion may be the presence of unabsorbed carbohydrates and lipids, but not amino acids within the lumen of the ileum (Holst, 1996). Glucagon like peptide-1 is released in monogastrics from the distal ileum and large intestine (Holst, 1996) and absorption of digested nutrients could be linked to GLP-1 secretion. The infusion of highly digestible nutrients into the

proximal small intestine may not have delivered a sufficient load to the ileum to stimulate GLP-1 release.

The present findings that dietary components reaching the intestine do not alter CCK release in the bovine are in agreement with Furuse et al., (1991b). Furuse et al (1991b) attributed the lack of effect of feeding concentrate and hay on plasma CCK concentration to the continuous and constant flow of digesta from the rumen and abomasum into the small intestine.

An additional explanation for the lack of response of circulating CCK concentration to the short term nutrient fluxes of both our present study as well as Furuse et al. (1991b) may be that the ruminant intestinal responses occur at a slower pace than in the monogastric. In effect a prolonged change of diet would be necessary for a response to occur. Our previously reported change of CCK mRNA in response to fasting and refeeding represented the response to a drastic and prolonged change of dietary regime, and we observed that the CCK mRNA abundance returned to the pre-fasting values within 16 h of refeeding (Chapter 4).

Furuse et al. (1991a) demonstrated an increased CCK secretion one hour after the start of a duodenal bolus (2 mmol amino acid dissolved in 20 ml of saline) of phenylalanine or tryptophan in goats. Varying experimental design or different species may explain the difference in response, especially considering that a 2 mmol bolus may have been a non-physiologically high nutrient flux, compared to .38 mmol min^{-1} in our study. Choi et al. (1996) showed that a high fat diet increased the CCK plasma concentration and reduced feed intake in Holstein cattle; however, they measured the CCK plasma concentration after a 14 day adaptation period. Christensen et al. (1994) also reported on decrease in feed intake following duodenal infusion of long chain fatty acids in Holstein cattle, which could have been mediated by changes in CCK, although the blood CCK concentrations were not measured. Their study also included a 21 day adaptation period. Furuse et al. (1991a) failed to measure a change of blood CCK concentration within the first three hours after feeding in goats after a 7 day adaptation to diets with increased crude protein concentration.

Another possible explanation for the lack of change in CCK concentration is that there was an altered digesta viscosity of the duodenal content, which may have interfered with, or blocked a CCK secretion response. Husar et al. (1996) recently reported that intestinal viscosity influenced CCK concentration in portal blood of two mature sheep. Viscosity was altered in their experiment by infusion of saline, carboxymethyl cellulose (CMC) in saline, CMC containing casein, or CMC containing casein and tannin. Husar et al. (1996) showed that increased viscosity using CMC led to reduction in CCK concentration in portal blood. However, the doubling or tripling of the viscosity by the addition of casein and respectively casein and tannin, resulted in a further reduction in the blood concentration of CCK. Although we did not measure viscosity of the infusates, during our experiment we observed that the viscosity of phenylalanine-, casein-, and starch-infusates were higher than the other treatments. It is therefore possible that the stimulation of CCK release was mitigated by increased viscosity, which appears to reduce CCK release.

An alternative explanation for our findings is that the hepatic clearance and thus the turnover of CCK peptides changes in response to the altered dietary treatment. Mir et al. (1996) reported a correlation coefficient of .67 between jugular and portal CCK plasma concentration. Mir also found that the correlation coefficient increases with altered viscosity (personal communication). Differences in hepatic clearance of CCK peptides in response to dietary changes have not previously been investigated. In the present experiment, blood was obtained from the jugular vein to avoid the additional stress associated with placing catheters in the portal vein.

The lack of change in CCK and GLP-1 plasma concentration may reflect the lack of a role in humoral signaling in response to the supplemented nutrients, however, it remains to be determined whether these hormones have a role in paracrine signaling mechanisms in the small intestine. In addition, changes in post-translational processing, hormone turnover, receptor abundance, and (or) binding of the hormones to their respective receptors are mechanisms that still need to be explored before an involvement of either hormone in the mediation of nutritional changes can be excluded. The choice of

a Latin square design was made to help eliminate the animal as a source of variation. It is however, important to notice that the animal variation for the mRNA baseline sample from week to week was a big source of variation. For one heifer the CCK mRNA abundance in the baseline sample ranged for example from .47 to 1.28 OD units. The Latin square design helped to standardize animals on the basis of pre-treatment values, but it did not correct for day to day variation in the response of individual animals, which may have contributed to the lack of significant changes in CCK or GLP-1 release in response to the infusion treatments.

Although the findings of our present study do not support the hypothesis that short term infusions of specific nutrients into the intestine influence release of CCK or GLP-1 we cannot rule out the possibility of post-translational control of CCK and GLP-1 or their involvement in paracrine signaling mechanisms.

5.3.3 *Effect of Altered Intestinal Digesta Composition on Feed Intake*

The 24 h cumulative feed intake data on the day of infusion was not different from the average intake two days prior to and two days after the infusion for any of the infusion treatments (FIGURE 5.7 Analysis of the 2h, 4h, and 8 h feed intake data on the day of infusion revealed that there were no significant differences in response to any of the infusion treatments (FIGURE 5.8 A and B). In particular, for the oil infusion it was expected that there would be a change in the 2h or 4h feed intake value because Christensen et al. (1994) showed a reduction of feed intake in response to fatty acid infusion into the abomasum. Choi and Palmquist (1996) also reported that feed intake was decreased linearly by increasing amounts of fat in the diet. Choi et al. (1996) reported that CCK-receptor antagonists were able to attenuate the endogenous CCK-induced reduction in feed intake. However, all of these studies measured feed intake after an adaptation period of 14 to 21 days to the feeding regime. Thus a longer adaptation period to the dietary changes in our study may have revealed an effect. Farningham et al. (1993) reported that synergistic actions between circulating CCK and propionate concentration were necessary to produce a reduction in feed intake, whereas either

treatment alone failed to reduce feed intake. Therefore it is possible that both the level and duration of infusion in our study were not sufficient to exert an effect on feed intake.

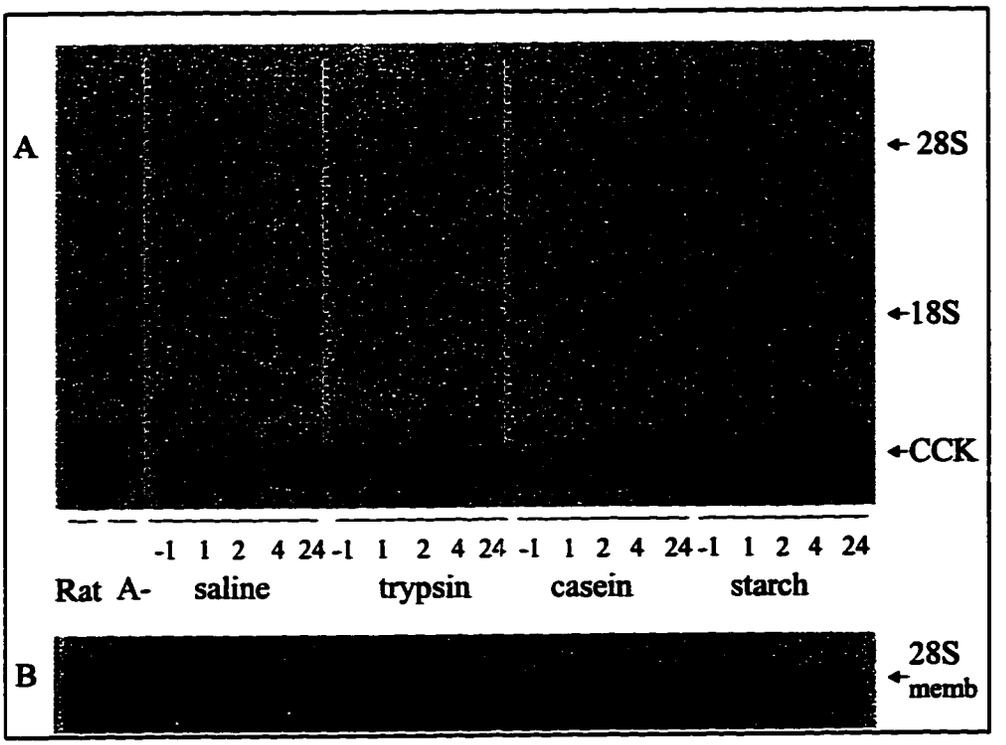


FIGURE 5.1 - Complete Autoradiogram of Northern Hybridization of RNA Isolated from Duodenal Mucosa Biopsies with CCK Riboprobe after Nutrient Supplementation by Infusion

Autoradiogram depicting the effect of nutrient supplementation by gastrointestinal infusion on CCK mRNA abundance of one heifer (A). Twenty micrograms of total RNA derived from duodenal biopsies taken at each sampling time from one heifer, 20 μg rat duodenum RNA (RAT; positive control), and 20 μg bovine duodenum RNA depleted of polyadenylated RNA (A^- ;negative control) are shown. The location of 28S and 18S rRNA and CCK mRNA are indicated. Autoradiographic exposure was 19 h. A photograph of the Northern transfer membrane is presented (B). Ethidium bromide stained 28S ribosomal RNA reveals equal loading of similar amounts of RNA in different lanes.

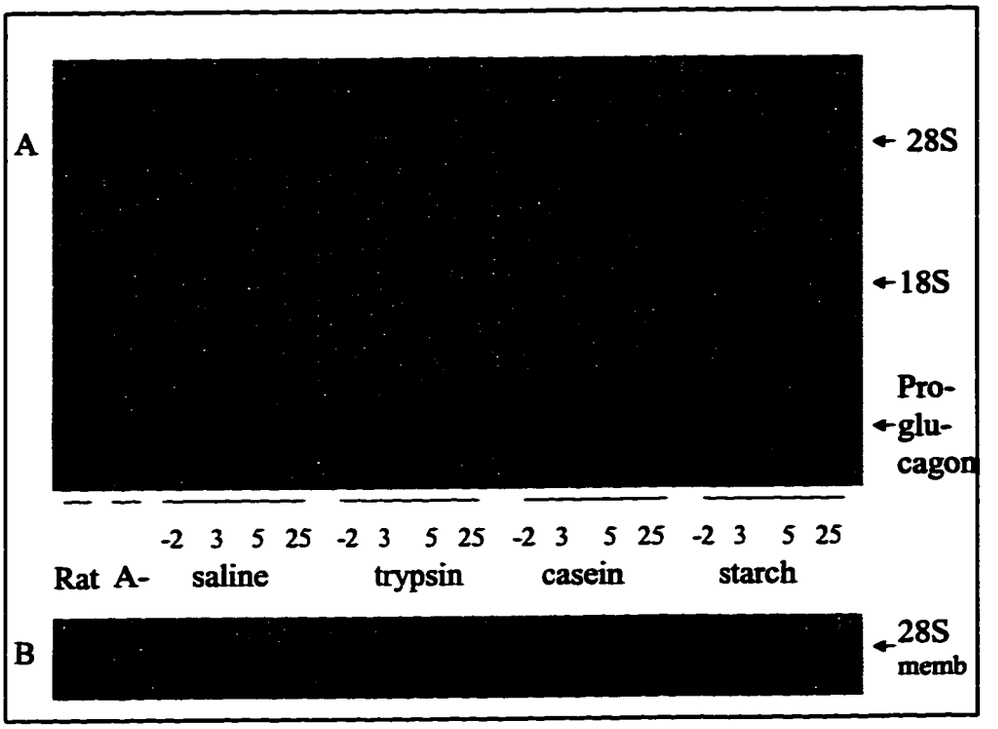
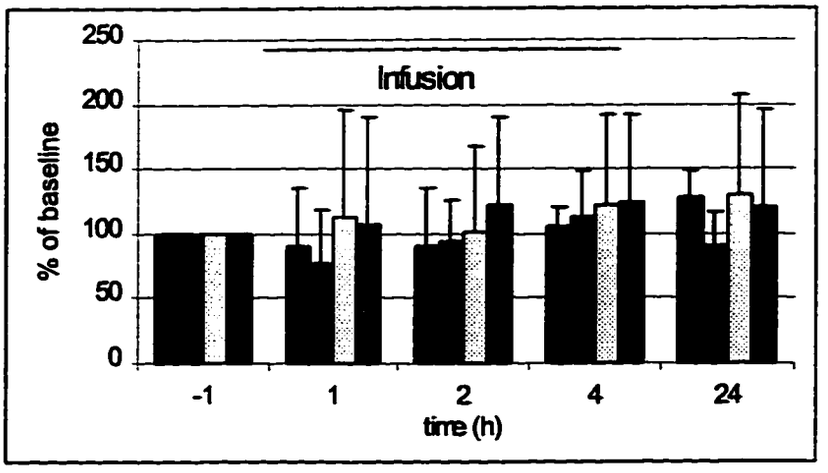


FIGURE 5.2 - Complete Autoradiogram of Northern Hybridization of RNA Isolated from Ileal Mucosa Biopsies with Proglucagon Riboprobe after Nutrient Supplementation by Infusion

Autoradiogram depicting the effect of nutrient supplementation by gastrointestinal infusion on proglucagon mRNA abundance of one heifer (A). The treatments were saline, trypsin, casein, and starch. Fifteen micrograms of total RNA derived from ileal biopsies taken at each sampling time from one heifer, 15 μg rat duodenum RNA (RAT; positive control), and 15 μg bovine ileum RNA depleted of polyadenylated RNA (A^-; negative control) are shown. The location of 28S and 18S rRNA and CCK mRNA are indicated. Autoradiographic exposure was 19 h. A photograph of the Northern transfer membrane is presented (B). Ethidium bromide stained 28S ribosomal RNA reveals equal loading of similar amounts of RNA in different lanes.



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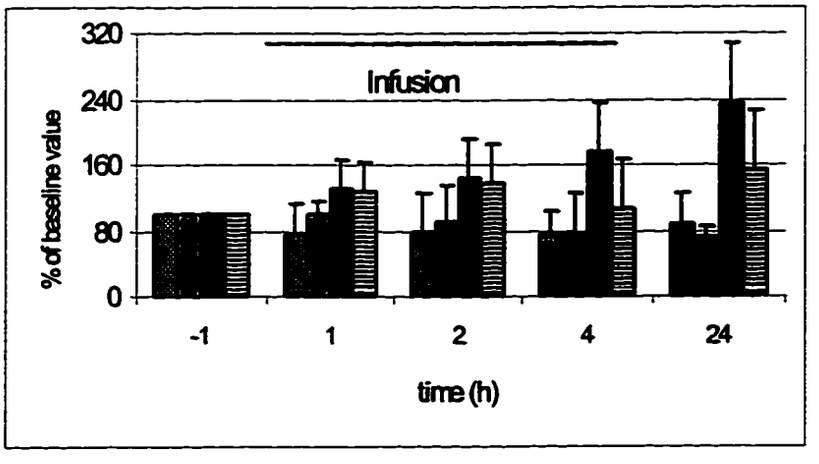
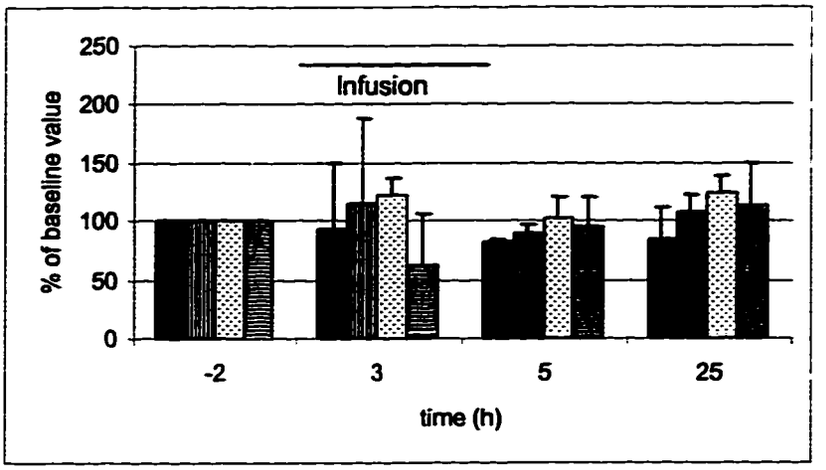


B

FIGURE 5.3 - Effect of Gastrointestinal Nutrient Supplementation on CCK mRNA Abundance in Bovine Duodenal Mucosa

Effect of 4 h abomasal infusion of SBTI (dark), canola oil (vertical lines), phenylalanine (light), or saline (horizontal) on the CCK mRNA abundance (A), and effect of 4 h duodenal infusion of trypsin (checker), or 4 h abomasal infusion of starch (diagonal lines), casein (black), or saline (horizontal), on CCK mRNA abundance of dairy heifers (B). Each sampling point is expressed as a percentage of the baseline value, which was derived from duodenal biopsy samples taken 1 h before the start of the infusion, to compensate for variability among animals in basal CCK mRNA abundance. The CCK mRNA abundance in the biopsies taken from the same animal 1 h before the start of infusions ranged from .20 to .75 OD units in the first and from 1.09 to 2.87 OD units in the second Latin square. (Data points: mean; error bars: SD; n = 4).



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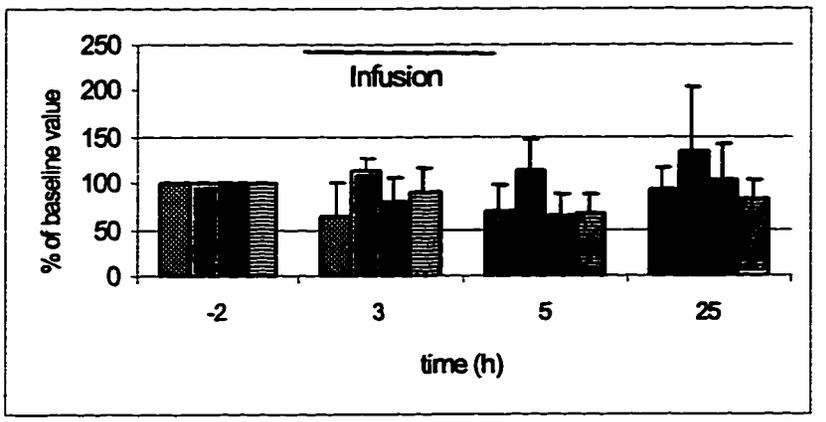
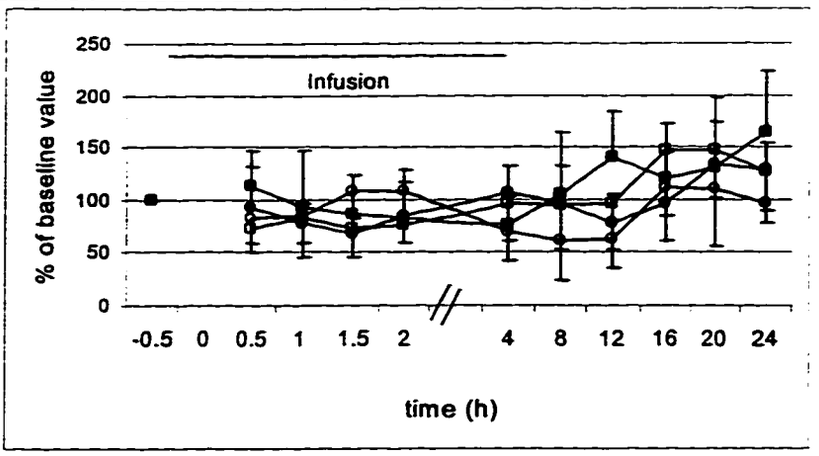


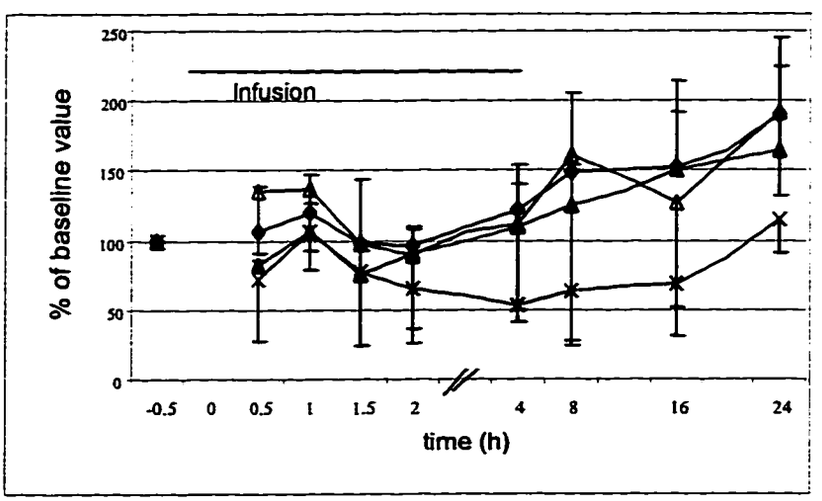
B

FIGURE 5.4 - Effect of Gastrointestinal Nutrient Supplementation on proglucagon mRNA Abundance in Bovine Ileal Mucosa

Effect of 4 h abomasal infusion of SBTI (dark), canola oil (vertical lines), phenylalanine (light), or saline (horizontal) on the proglucagon mRNA abundance (A), and effect of 4 h duodenal infusion of trypsin (checker), or 4 h abomasal infusion of starch (diagonal lines), casein (black), or saline (horizontal), on proglucagon mRNA abundance of dairy heifers (B). Each sampling point is expressed as a percentage of the baseline value, which was derived from ileal biopsy samples taken 2 h before the start of the infusion, to compensate for variability among animals in basal proglucagon mRNA abundance. The proglucagon mRNA abundance in the biopsies taken from the same animal 1 h before the start of infusions ranged from 1.51 to 3.76 OD units in the first and from .93 to 2.22 OD units in the second Latin square. (Data points: mean; error bars: SD; n = 3).



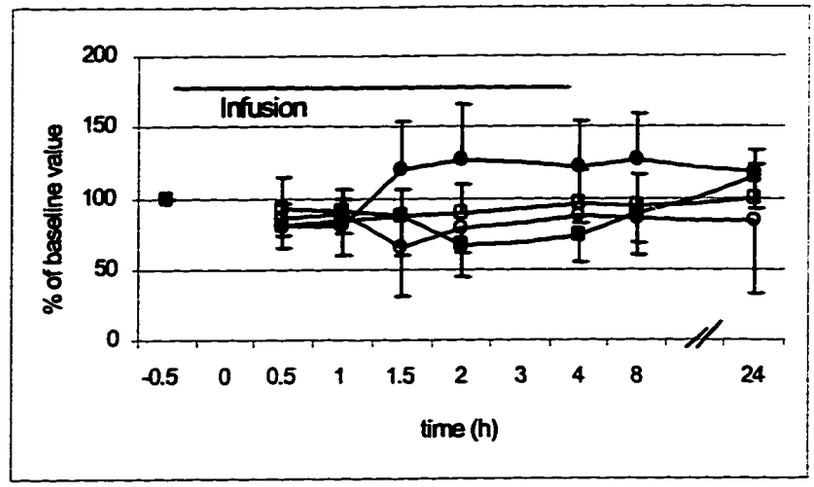
A



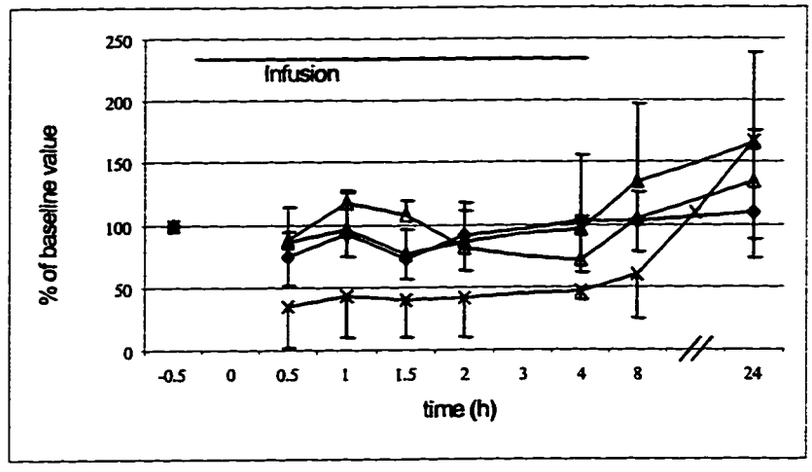
B

FIGURE 5.5 - Effect of Gastrointestinal Nutrient Supplementation on Bovine CCK Blood Concentration

Effect of 4 h abomasal infusion of SBTI (■), canola oil (□), phenylalanine (•), or saline (o) on the CCK blood concentration (A), and effect of 4 h duodenal infusion of trypsin (▲), or 4 h abomasal infusion of starch (Δ), casein (♦), or saline (×), on CCK blood concentration of dairy heifers (B). Each sampling point is expressed as a percentage of the baseline value, which was derived from blood samples taken 1 h before the start of the infusion, to compensate for variability among animals in basal CCK blood concentration. The CCK blood concentration in the samples taken from the same animal 1 h before the start of infusions ranged from 5.43 to 13.6 pmol l^{-1} in the first and from 10.8 to 22.8 pmol l^{-1} in the second Latin square. (Data points: mean; error bars: SD; n=4).



A



B

FIGURE 5.6 - Effect of Gastrointestinal Nutrient Supplementation on Bovine GLP-1 Blood Concentration

Effect of 4 h abomasal infusion of SBTI (■), canola oil (□), phenylalanine (•), or saline (○) on the GLP-1 blood concentration (A), and effect of 4 h duodenal of trypsin (▲), or 4 h abomasal infusion of starch (Δ), casein (♦), or saline (×), on CCK blood concentration of dairy heifers (B). Each sampling point is expressed as a percentage of the baseline value, which was derived from blood samples taken 1 h before the start of the infusion, to compensate for variability among animals in basal GLP-1 blood concentration. The GLP-1 blood concentration in the sample taken from the same animal 1 h before the start of infusions ranged from 69.1 to 196.6 pmol l^{-1} in the first and from 61.1 to 257.8 pmol l^{-1}in the second Latin square.(Data points: mean; error bars: SD; n=4).

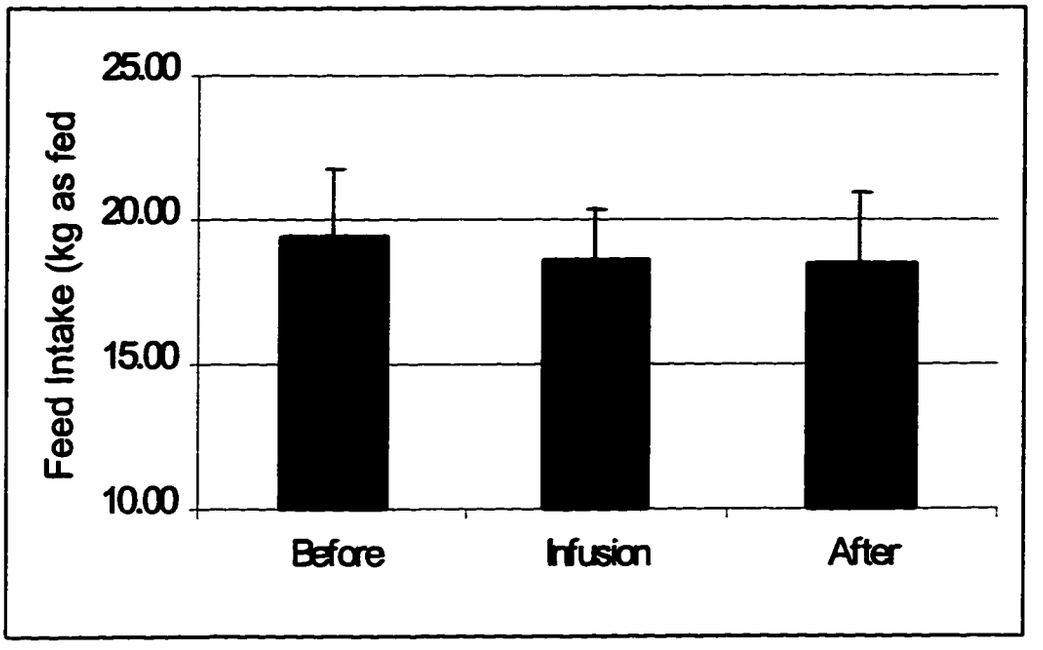


FIGURE 5.7 - Effect of Gastrointestinal Infusion on Daily Feed Intake

Effect of gastrointestinal infusions on the cumulative feed intake of dairy heifers. The average 24 h feed intake data two days before, two days after and on the day of infusion are shown. (Bars: means; error bars: SD; n = 4)

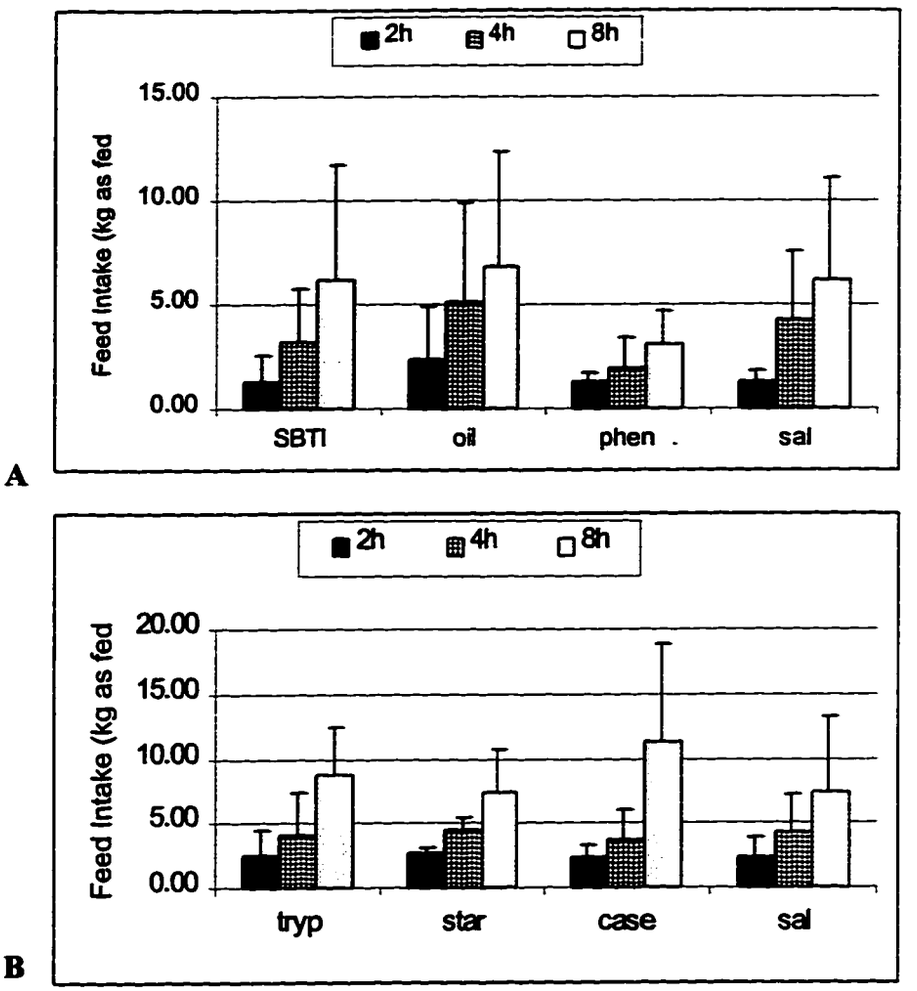


FIGURE 5.8 - Effect of Gastrointestinal Nutrient Supplementation on 2, 4, and 8 h Feed Intake

Effect of 4 h abomasal infusion of SBTI, canola oil (oil), phenylalanine (phen), or saline (sal) on the cumulative feed intake (A), and effect of 4 h duodenal infusion of trypsin (tryp), or 4 h abomasal infusion of starch (star), casein (case), or saline (sal), on cumulative feed intake of dairy heifers. The average 2, 4, and 8 h feed intake data measured by automatic feed scales are shown (Bars: means; error bars: SD; n = 4).

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Chapter 6 - General Discussion and Conclusion

6.1 - Introduction

Recent interest in intestinal satiety signals in the ruminant has occurred following evidence that control of feed intake cannot solely be explained on the basis of feed characteristics, ruminal control of fill, and mean retention time (Mathison et al., 1995). Increased feed intake during periods of increased physiological demand, e.g. peak lactation (Baile and Della-Fera 1988), following somatotropin administration (Burton et al., 1994), cold environmental temperature (Kennedy et al., 1986), or decreased feed intake following intra-abomasal infusion of long chain fatty acids (Christensen et al., 1994) are indications that satiety cues regarding physiological state and (or) absorbed nutrients are utilized by ruminants to alter feed intake. Although the primary function of cells in the gastrointestinal tract is considered to be nutrient absorption, they also play key roles in the endocrine and neural network by responding to changes in nutrient supply as part of an integral feedback system that ensures adequate intake for maintenance, lactation and growth. Current knowledge about gastrointestinal satiety signals in ruminants is limited.

CCK is one of the gastrointestinal neuropeptides which is released in response to nutrients, in particular protein and lipids, reaching the intestine of monogastric species (Rehfeld, 1989; Ritter et al., 1994). A large body of evidence has accumulated demonstrating that CCK impacts feed intake in several monogastric species (Gregory et al., 1989; Liddle et al., 1988; Smith and Gibbs, 1994) as well as in sheep (Della-Fera et al., 1981). Proglucagon is the gene encoding for several gastrointestinal peptides, e.g. GLP-1 and GLP-2, released from the distal small intestine, which are involved in the regulation of pancreatic endocrine secretion (Holst et al., 1987), gastric acid secretion and gastric emptying (Wettergren et al., 1993 and 1994). A role for GLP-1 in central regulation of feeding has also been suggested (Turton et al., 1996). Although there are some studies that have investigated the role of GLP-1 in sheep (Martin and Faulkner,

1993, and 1996), the presence and role of the proglucagon gene and its products in the bovine have not been established.

In order to increase our understanding about intestinal satiety signals and their involvement in ruminant feed intake regulation we studied CCK gene and protein and the proglucagon gene and GLP-1 peptide as candidates to be released in response to nutrient reaching the intestine. Changing the amount and composition of digesta reaching the small intestine by fasting and refeeding, and by gastrointestinal supplementation of nutrients were used to investigate the role of intestinal satiety signals in feed intake regulation.

6.2 - CCK Radioimmunoassay

Radioimmunoassays measure the concentration of biologically active peptide produced following transcription, translation and post translational processing events in responding intestinal cells. Several RIA (Hashimura et al., 1982; Jansen and Lamers, 1983; Beardshall et al., 1992) have been used successfully and have helped to accumulate information regarding the role of CCK in regulating feed intake in monogastrics. In ruminant species only a few studies have measured CCK plasma concentrations (Furuse et al., 1991; Furuse et al., 1992; Guilloteau et al., 1992; Zabielski, et al., 1992; and Choi and Palmquist, 1996).

In order to measure circulating CCK concentrations in bovine blood during the current study, a radioimmunoassay was validated in our laboratory. Extraction of CCK peptides from plasma by either C^{18} Sep-Pak cartridge chromatography or ethanol extraction was necessary to avoid nonspecific interference of hydrophilic plasma fractions on the measurement of CCK concentration by RIA. Parameters measured to control the accuracy of each RIA (non-specific binding, percentage specific bound, estimated dosage 20, 50, and 80, detection limit of the assay) were very similar for both extraction methods. However, the C^{18} Sep-Pak cartridges used during the validation appeared to have a higher variability of extraction efficiency than the ethanol extraction method. In addition, the ethanol extraction method was logistically easier to use during

our experiments due to the large number of blood samples requiring analysis within a short period of time. Thus, the data presented in this thesis are from samples extracted using the ethanol extraction method. CCK blood concentration data obtained does not allow the analysis of possibly post-translational processing changes leading to an altered distribution of CCK-peptides with varying length, due to the fact that the Deno antibody binds all biologically active CCK peptides equally.

6.3 - In Vivo Method to Study Nutrient-Gene Interaction

In monogastrics nutrient-gene interactions relating to feed intake regulation have been researched intensively (Liddle et al., 1988; Docherty and Clark, 1994). Measurements of specific mRNA species provide insight into factors which modulate the transcription of genes, a prerequisite to translation of genetic information into proteins which may in turn cause functional changes in an organism. Methods to measure RNA abundance (or RNA steady state) previously described by Glimm (1991) were optimized to detect CCK and proglucagon mRNA abundance (Appendices 1 to 5). The CCK cDNA fragment was subcloned (Appendix 1.1) into plasmid pBluescript®II SK, plasmid DNA was isolated (Appendix 1.2), and linearized (Appendix 1.3) to allow the use of RNA polymerases. The proglucagon cDNA was available in pGEM plasmid, which is a vector also designed for *in vitro* transcription reaction. Total and polyadenylated RNA was isolated from bovine duodenal mucosa tissue (Appendices 2.1 to 2.3) which has been collected postmortem at a local abattoir or isolated from rat duodenal mucosa tissue. Northern blot analysis was performed to determine the size and abundance of the CCK and proglucagon mRNA (Appendices 3.1 to 3.4) using a CCK and proglucagon riboprobe. It was concluded that CCK mRNA abundance in bovine duodenal mucosa was at the limit of detection in total RNA preparations by Northern blot analysis, whereas CCK mRNA in the rat duodenum could easily be detected (FIGURE 3.1). Consequently more sensitive assay procedures such as ribonuclease protection assay and the cloning of the bovine CCK mRNA by polymerase chain reaction (PCR) were pursued in order to improve the detection of CCK mRNA in total RNA isolated from bovine duodenum (Appendices 4 and 5). However, the development of a duodenal biopsy procedure using

cannulated animals allowed the measurement of CCK mRNA abundance by Northern blot analysis in as little as 15 to 20 μg total RNA (FIGURE A3.1). As a result Northern blot analysis was applied in further studies, which also proved to be appropriate for detecting proglucagon mRNA in ileal biopsy tissue. The isolation of RNA using either the GIT-CsCl or TRIzol® method was found to give comparable results on intestinal biopsy tissue (FIGURE A3.1). We used the easier TRIzol method to isolate RNA during the development of methods to allow the investigation of nutrient gene interactions.

Historically, animals had to be sacrificed in order to obtain gastrointestinal tissues (cells) needed to evaluate the influence of nutrition on gene expression, in particular research relating to function or metabolism of enterocytes (Okine et al., 1994). The development and improvement of molecular biology methods (i.e., Northern hybridization analysis using a minute amount of RNA) makes it much easier for scientists to investigate nutrient-gene interactions (Greenstein et al., 1990; Liddle et al., 1988; Kanayama and Liddle, 1991). In rats or mice, animal variation in mRNA abundance can be counteracted by the use of a large number of animals per treatment, because the cost of the animals is a minor factor. Dairy cows are not only costly, but also belong to a genetically more heterogeneous population than laboratory rodents, which makes large differences in mRNA abundance between animals a common phenomenon (Zhao, 1995; Beswick, 1997).

Studies with rumen papillae (Kelly et al., 1991; Harmon, 1986), mammary biopsy tissue (Glimm et al., 1992), mammary fat pad tissue (Hovey et al., 1996), mammary tissue explant culture (Yang et al., 1997), subcutaneous adipose biopsy with adipocyte explant cultures (Vazquez-Añón et al., 1996), and liver biopsy to obtain hepatocytes monolayer cultures (Strang et al., 1996) are some techniques used to study physiological changes or changes in gene expression without having to sacrifice large ruminants.

Intestinal cannulation of cows together with fiberoptic endoscopy allows easy access to intestinal mucosa without having to sacrifice the animal as demonstrated by Sterzing et al. (1971) to study lipid absorption. Enterocytes have a very short life-span (Shirazi-Beechey, 1995), which makes tissue culture studies on their metabolism

difficult. However, this can be seen as an advantage for the intestinal biopsy procedure because the intestinal mucosa is a tissue with a very large surface area and immense regeneration capacity, which allows for frequent sampling without complications for the animal.

We evaluated the effectiveness of the intestinal biopsy sampling method as a tool to study nutrient-gene interactions. This evaluation revealed the usefulness of the method for studying the influence of nutrients reaching the small intestine on expression of gastrointestinal genes and possible implications for feed intake regulation. An additional advantage of monitoring the response to a treatment within one animal is that the variation between animals can be eliminated if the results are expressed as relative changes compared to a baseline value. However day to day variations in mRNA abundance appear to be high, so that the baseline values differ much from one experimental period to the next within the same animal.

6.4 - Effect of Feed Restriction and Different Physiological States on CCK Gene Expression and CCK Release in Ruminants

To test our hypothesis that nutrients reaching the intestine would induce the release of gastrointestinal hormones such as CCK which could then serve as satiety signals, we evaluated the effect of feed restriction and physiological state on CCK mRNA abundance and CCK release in ruminants. Intestinal tissue was collected after slaughter at a local abattoir. Prior to slaughter three non-lactating and four lactating cows were fed total mixed rations to meet their requirements. The other lactating animals were fasted for three days prior to slaughter.

The blood CCK concentrations measured three days before, and on the day of shipment to the abattoir revealed that the fasted cows had a lower blood CCK concentration after feed deprivation than the fed animals. No difference in blood CCK concentration was detected between lactating and non-lactating cows. Fasting reduces nutrient supply to the small intestine which was predicted to reduce CCK gene expression and CCK synthesis. Our analysis of blood CCK concentrations confirmed this hypothesis

which is consistent with data reported in fasted and refed rodents (Greenstein et al., 1990; Kanayama and Liddle, 1991)

A low CCK mRNA abundance was demonstrated in the duodenal tissue of all cows. In jejunum and ileum tissue, a CCK mRNA signal was detectable in 40 μg total RNA though at a lower level than in duodenal tissue. In rumen tissue collected postmortem, CCK mRNA was detected in 20 μg A^+ RNA, but not in 40 μg total RNA. The weak signal of CCK mRNA and the magnitude of the individual differences together with the small number of animals are likely reasons for the lack of detectable significant differences in CCK mRNA abundance between the fasted and fed as well as between lactating and non-lactating cows. At the time of the blood sampling the animals were still in their accustomed environment and the expected blood CCK concentration response could be observed. We therefore concluded that stressors (e.g., shipment, unfamiliar environment and handlers) associated with slaughter at the abattoir contributed to the lower abundance of CCK mRNA observed in all post-mortem tissue samples. However, we did not test the effect of the time from the animal's death to the tissue collection on the steady state of mRNA in a controlled experiment to determine the CCK mRNA half life time. Considering that the RNA steady state depends not only on the transcription of the genes but is also determined by the RNA half life times (Ross 1996) this information will in future help to determine the effect of stress on CCK mRNA abundance.

6.5 - Longitudinal Distribution of CCK mRNA in the Ruminant Digestive Tract

In the monogastric gastrointestinal tract, CCK is known to be most abundant in the proximal small intestine (Rehfeld 1989). Information about relative mRNA abundance throughout the ruminant gastrointestinal tract has not been documented.

To determine the longitudinal distribution of CCK gene expression in the bovine gastrointestinal tract two lactating dairy cows were killed at the research facility, and mucosa (abomasum, duodenum, jejunum, and ileum) and tissue (rumen and omasum) samples were taken, pooled, snap frozen as described above within 30 min of the animal's death, and analyzed for CCK mRNA abundance.

Cholecystokinin mRNA was demonstrated to be most abundant in the proximal small intestine, but was also detectable in jejunum and ileum in 5 μg A^+ RNA and in 30 μg total RNA. In the other gastrointestinal tissues including the rumen, no CCK mRNA signal could be detected in 5 μg A^+ RNA. The tissues that were collected after slaughter at the research facility showed a relatively higher CCK mRNA abundance than the postmortem tissues collected at the local abattoir. The intestinal biopsy method was judged to be superior to either of the post-mortem tissue collection methods, which is likely due to the minimal stress associated with this procedure as well as the reduced time from severance from the mucosal tissue until the freezing of the biopsies. The intestinal biopsy method was chosen for the remaining studies reported in this thesis.

6.6 - Effect of Fasting and Refeeding on the CCK and Proglucagon Gene and Feed Intake

We investigated the hypothesis of dietary regulation of CCK and proglucagon gene expression as well as CCK and GLP-1 release in the bovine small intestine during a 48 h period of fasting and refeeding, which would be characteristic of a physiological satiety effect of a gut hormone (Smith, 1984).

The main observations were that CCK mRNA abundance, CCK blood concentration, and GLP-1 blood concentration decreased during the period of fasting. However, by 8 to 36 h after refeeding all values had increased to the point where they were not statistically different from pre-fasting values.

The fasting and refeeding-induced changes in abundance of CCK mRNA were paralleled by similar changes in CCK blood concentration. Findings of reduced CCK mRNA following food deprivation and recovery to pre-fasting values are in agreement with several studies in rats (Koop et al., 1987; Greenstein et al., 1990 and Kanayama and Liddle, 1991). In contrast, there were no detectable changes in the abundance of proglucagon mRNA even though fasting and refeeding induced changes in the blood concentration of GLP-1 similar to those seen for CCK, indicating that there are

substantial differences in the molecular mechanisms underlying the regulation of CCK and GLP-1. For a gastrointestinal hormone to fulfill any function related to regulating the digestion of food or controlling hunger or satiety, its gene would have to be transcribed, its mRNA translated, and the protein synthesized, modified, and released. Presumably nutrient interaction with gastrointestinal cells would influence one or more of these regulatory processes. The changes observed for CCK and GLP-1 plasma concentrations indicate that both of these gastrointestinal hormones play a role in regulating food digestion and possibly controlling feed intake in ruminants.

We also observed that the short term food intake of the animals was not changed by reduced plasma concentrations of CCK or GLP-1 following the 48 h fasting period. The 24 h intake on the first day following the fasting period was reduced possibly through other feed intake limiting mechanisms, such as lack of normal rumen distention during fasting and (or) a reduced rumen microbial population at the start of the refeeding period. These observations indicate that after dramatic changes such as 48 h fasting the nutrients reaching the small intestine are not the only satiety inducing mechanisms in ruminants.

Our hypothesis that changes induced by nutrients reaching the small intestine such as the release of the gastrointestinal hormones CCK and GLP-1 are partly responsible for the regulation of feed intake in ruminants, appears to be confirmed by the results of this study.

6.7 - Effect of Gastrointestinal Nutrient Supplementation on CCK and Proglucagon Gene and Feed Intake

Once it was determined that CCK and GLP-1 were released in response to nutrients reaching the small intestine in fasted animals we evaluated the role of intestinal satiety factors in fed ruminants.

In different species, specific nutrients and nutritional factors have been shown to induce or inhibit CCK release. In ruminants, high fat diets have been shown to increase

plasma CCK and reduce feed intake in lactating cows (Choi and Palmquist, 1996) and duodenal infusions of phenylalanine increased plasma CCK concentrations in goats (Furuse et al., 1991). Synergism was shown to exist between propionate and CCK in the control of feed intake in sheep (Farningham et al., 1992). Glucagon-like peptide-1 is the major intestinal proglucagon product in monogastric species (Ørskov et al., 1986). Although there are studies which have investigated the role of GLP-1 in sheep (Martin and Faulkner, 1993 and 1996), the presence of GLP-1 and nutrients that cause its release in the bovine have not been reported.

The effect of abomasal infusion of nutrients or soybean trypsin inhibitor (SBTI), or duodenal infusion of trypsin in fed animals on the regulation of CCK and proglucagon gene expression during two 4 x 4 Latin square experiments were investigated.

CCK mRNA abundance, CCK blood concentrations, proglucagon mRNA, and GLP-1 blood concentrations were not different between animals infused with SBTI, oil, phenylalanine, or saline in the first Latin square, or for the animals infused with casein, starch, trypsin, or saline in the second Latin square. For the phenylalanine infusion there was a trend towards increased GLP-1 release after 1 h 30 min ($p=0.069$) and 2h ($p=0.091$), but none of the treatments induced significant changes in GLP-1 release, CCK release, proglucagon, or CCK mRNA abundance.

The lack of change in mRNA abundance, however, is not sufficient to exclude a change of CCK or proglucagon peptides being produced in response to specific nutrient supplementation. The possibilities of modified translation, mRNA turnover, post-translational processing, protein turnover, receptor binding, or number of receptors in response to nutrient fluxes would have to be eliminated before a lack of treatment effect can be verified. Moreover, the earliest response in proglucagon mRNA change was shown in rats after 6 h (Tappenden et al., 1997) thus it may be that the duration of infusion and (or) delivery of nutrients to the ileum was insufficient to increase proglucagon gene expression in ruminants. Liddle et al. (1988) demonstrated in rats that an increased plasma CCK concentration in response to duodenal trypsin inhibitor infusion was followed not only by increased mRNA abundance, but was also

accompanied by an increase in CCK gene transcription which reached a maximum within 4 h. Shirazi-Beechey (1995) indicated that the time needed for complete cell renewal of enterocytes in the intestinal lumen varies between species from 2 to 6 days and these species differences could contribute to a differential response in gastrointestinal gene expression to nutrient fluxes in ruminants relative to rodents. Prolonged infusions of specific nutrients into the abomasum of cattle may therefore be necessary to induce the changes in gastrointestinal hormone mRNA abundance observed with short-term nutrient infusions in monogastrics. Research on the changes in transcription and mRNA turnover will be required to fully understand the processes initiated to alter mRNA steady state of gastrointestinal hormones in response to dietary changes.

Our findings that neither CCK nor GLP-1 release was altered appear to contradict previous reports of phenylalanine, fat, or soybean trypsin inhibitor stimulating CCK secretion in ruminants (Furuse et al., 1991; Choi and Palmquist, 1996; and Kato et al., 1986). However the varying experimental designs and species may explain the difference in response, in particular with respect to the duration of the nutritional supplementation. Altered hepatic clearance of CCK peptides (Mir et al., 1996) may be a factor in reducing the response in CCK secretion before it is measured at the jugular vein.

The lack of changes in CCK or GLP-1 plasma concentration may be a negative indication for a role of these peptides in humoral signaling in response to the supplemented nutrients. Their role in paracrine signaling mechanisms in the small intestine, and subsequent changes in post-translational processing and receptor changes still need to be explored before an involvement of either peptide in the mediation of nutritional changes can be excluded.

We found that no significant difference in short term or daily feed intake occurred as a response to any of the infusion treatments, which contradicts findings of a decrease in feed intake in dairy cattle after lipid supplementation (Choi and Palmquist, 1996; and Christensen et al., 1994). However, both of these studies involved a longer adaptation period to the dietary regime, which may be an indication that the duration and level of infusion in our study were not sufficient to exert an effect on feed intake.

Further research will be needed to confirm whether longer alterations in dietary composition, e.g. by prolonged infusion and (or) increased magnitude of nutrient flux, can induce changes in the release of CCK and GLP-1. The influence of nutrients in combination with changes of volatile fatty acids absorbed from the rumen would be of interest considering findings in sheep of Farningham et al. (1992). The involvement of CCK and GLP-1 in paracrine signaling pathways, post-translational changes, and changes in their receptors will have to be investigated to confirm their involvement in the regulation of feed intake in the ruminant. In particular the possibility that the hepatic clearance of CCK peptides might change in response to the altered dietary treatment (Mir et al., 1996), would have to be addressed in future studies by collection of blood at the portal vein in addition to the jugular vein. Ideally, a blood sampling system should be used that avoids disturbing the animal at every sampling time. Animal variation could be reduced by involving only gestating cannulated animals in the study to avoid interference of gonadotrophic and other hormones with feeding behavior. The expansion of the '4 x 4' to a '5 x 5' or '6 x 6' Latin square design may also reduce the impact of 'between animal' and 'within animal-between period' variation.

6.8 - Conclusion

The results obtained in the presented experiments suggest that the biopsy sampling technique is an ideal method to study nutrient gene interaction in the ruminant. It was demonstrated that the gastrointestinal hormones CCK and proglucagon are released partially in response to nutrients reaching the small intestine in the fasted and refed bovine. A change of CCK mRNA could also be shown in response to fasting and refeeding. The findings in the study involving gastrointestinal supplementation of nutrients in the fed ruminant did not confirm our hypothesis that CCK and proglucagon are released as a response to short term nutrient flux in the small intestine. Further research will be needed to test whether prolonged duration of infusion and (or) higher inclusion levels of nutrients induce changes in the gastrointestinal hormone gene expression or release, and whether this impacts food intake regulation in the ruminant.

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Appendix Methods

Appendix 1 - Subcloning of cDNA Fragment, Purification and Linearization for Riboprobe Preparation

1.1 - *Subcloning in Low Melting Point Agarose*

Modified from Glimm, D.R. 1991. Role of Growth Hormone in Mammary Biology. PhD Thesis. University of Alberta. Appendix 1: p155-159.

Solutions for Subcloning Procedures:

LB liquid medium:
to make 500 ml:
to 450 ml of Milli Q (MQ) H_2O add:

Pepton	5.0 g
yeast extract	2.5 g
NaCl	5.0 g.

Use a magnetic stirbar until the solids have dissolved and then adjust pH to 7.0 with 5 N NaOH. Adjust the volume to 500 ml and autoclave.

Ampicillin stock:
Dissolve 50 mg of ampicillin in 1 ml of water.

LB-agar-ampicillin plates:
Make up 1 L of LB and add 15 g of agar. Autoclave. After autoclaving, let the solution cool to 65°C then add ampicillin stock solution (50 mg/ml) to the LB-agar to final concentration of 50 μg/ml. Pour the plates carefully. This volume allows for 20 - 24 plates to be poured. These plates can be stored for up to 30 days at 4°C.

Mix #1

100 mM NaCl
5 mM Mg Cl_2
5 mMTris-HCl, pH 7.6

to make 150 ml:

3 ml 5 M NaCl
.75 ml 1 M Mg Cl_2
.75 ml 1 M Tris-Hcl, pH 7.6.

Dissolve and bring to 150 ml with MQ H_2O then autoclave for 20 min.

Mix #2

100 mM CaCl
250 mM KCl
5 mM Mg Cl_2
5 mMTris-HCl, pH 7.6

to make 150 ml:

2.2053 g $CaCl_2$
2.796 g KCl
.75 ml 1 M Mg Cl_2
.75 ml 1 M Tris-Hcl, pH 7.6.

Dissolve and bring to 150 ml with MQ H_2O then autoclave for 20 min.

Preparation of DH∝ competent cells:

1. Start overnight bacterial culture by inocculating a single colony of DH∝ (E. coli) into 10 ml of Luria Bertani (LB) medium and grow overnight at 37 °C with shaking.
2. Add 500 μl of DH∝ overnight culture to 100 ml sterile LB.
3. Incubate at 37°C with shaking (200 rpm) for 2.5 h.
4. Aliquote LB containing DH∝ into 2 sterile 50 ml Oakridge tubes.
5. Centrifuge at 4000 rpm for 5 min (4°C) with no brake.
6. Decant supernatant.
7. Resuspend pellet in one tube in approx 45 ml of Mix #1.
8. Pour the resuspension mixture into the second tube and resuspend the second pellet.
9. Let stand on ice for 5 min.
10. Centrifuge at 4000 rpm for 5 min (4°C) with no brake.
11. Decant supernatant.
12. Add 20 ml Mix #2 and resuspend pellet.
13. Let stand on ice for 40 min.
14. Centrifuge at 4000 rpm for 5 min (4°C) with no brake.

15. Decant supernatant, resuspend pellet in 2 ml Mix #2.

16. Aliquot into sterile eppendorf tubes, 200 μl in each.

17. Allow to sand overnight at 4°C for optimum transformation efficiency and use fresh..

Alternatively the last pellet (step 15) can be resuspended in 8.6 ml of 0.1 M CaCl and 1.4 ml of glycerol, aliquoted into sterile microcentrifuge tubes, incubated overnight at 4°C, snap frozen in dry ice/alcohol, and stored at -76°C.

Transformation of plasmid containing the probe cDNA fragment into DH$\propto$ competent cells

1. Resuspend pellet plasmid DNA in 10 μl of 10 mM Tris HCl /0.1 mM EDTA. (puc 13 plasmid DNA containing CCK fragment was received precipitated)

2. Aliquot 100 μl of ice cold competent cells into cold 4 ml polypropylene tube
(If frozen cells are used they need to be thawed on ice very slowly!).

3. Add 5 μl of puc 13 plasmid to competent cells;
also add 1 μl of the desired vector plasmid DNA to competent cells (pBluescipt SK+).

4. Incubate on ice for 30 min.

5. Heat shock cells for 45 sec at 42°C.

6. Incubate at 37°C for 1 h.

7. Add 100 μl LB.

8. Place 200 μl on LB-agar-ampicillin plates and use sterile glastool to distribute evenly on plate.

9. Incubate overnight at 37°C only the ones with plasmid DNA will be able to grow onampicillin plates.

10. Wrap the plate with parafilm and store at 4°C. The colonies can be maintained by re-plating on LB-agar ampicillin plates every 30 days.

Amplification and isolation of plasmid DNA

1. Incubate 10 ml LB medium with single colony of DH∝ E. coli bacteria containing the puc 13 plasmid and 10 ml LB medium with a single colony of DH∝ E. coli bacteria containing the pBluescript plasmid and grow overnight at 37°C with shaking.

2. Isolate plasmid DNA, using plasmid purification kit (Sigma Chemical Compani, St. Louis, MO, USA). The puc 13 and pBluescript SK+ DNA was eluted using 50 μl of sterile water.

Digestion of plasmid DNA

1. Digestion of insert DNA
   11μl H_2O
   5 μl Insert DNA (puc 13 with CCK fragment)
   2 μl 10 x reaction buffer
   1 μl restriction enzyme #1 (Hind III)
   1 μl restriction enzyme #2 (SacI).

2. Digestion of vector DNA
   11μl H_2O
   5 μl vector DNA (pBluescript SK+)
   2 μl 10 x reaction buffer
   1 μl restriction enzyme #1 (Hind III)
   1 μl restriction enzyme #2 (SacI).

3. Incubation of reaction mix at 37°C for 1 h in water bath.

4. The reaction buffers needed for HindIII and SacI were not compatible, because the salt concentrations needed for optimal digestion efficiency were different. As a result the plasmids were first digested with SacI and Buffer A (lower salt concentration), then a phenol chloroform extraction was performed and followed by a digestion with HindIII and Buffer B.
   The CCK - fragment had been inserted into the puc 13 plasmid in J. Dixons's laboratory using HindIII and EcoRI. However a region of the pBluescript multiple cloning site (SMAI site with 9 nucleotide GGGGGCCG) which has been reported to increase non-specific background by binding to 28S rRNA during in situ hybridization (Witkiewicz et al., 1993. Bio Techniques 14:458) could be eliminated by digesting the pBluescript and puc 13 plasmids with HindIII and SacI restriction enzymes.

5. When digestion is completed add 2 μl of gel tracking dye to each reaction mixture.

6. Digestion fragments are separated in 1% low melt agarose gel. The DNA is stained with Ethidium Bromide. Load DNA, run at 100 volt for 10 min, then at 70 volt until

fragments of interest are resolved enough to excise.

7. Cut appropriate insert and vector fragments out of gel using a new sterile scalpel blade for each. Trim to get gel slice as small as possible.

8. Melt slices at 70°C for 5 - 15 min in water bath.

9. Combine an appropriate amount of vector and insert solution (2:1 to 4:1, insert: vector DNA) to give a final volume of 10 μl (e.g. 1μl vector gel, 9μl insert gel).

10. Allow mixture to equilibrate to 37°C for 15 min.

11. Add 10 μl ice-cold 2 x ligase buffer, containing T4 ligase, mix quickly.
    10 x ligase buffer:
    400 mM Tris-HCl, pH 7.5
    100 mM $MgCl_2$
    100 mM DTT
    10 mM ATP
    500 μg/ml BSA
    2 x buffer/T4 ligase
    15μl H_2O
    4μl 10 x ligase buffer
    1μl T4 ligase (BRL 1 unit/μl)

12. Incubate at 15°C overnight.

Transformation of ligated subclones into competent DH$\propto$ cells

1. Remelt 20 μl ligation reaction at 70°C for 15 min.

2. Aliquot 200 μl competent DH$\propto$ cells into cold 4 ml tubes.

3. Add 5 μl ligation reaction to competent cells.

4. Gently mix tubes and place on ice for 30 min.

5. Place LB-agar-ampicillin plates into 37°C incubator to warm.

6. Place transformation reaction tubes in 42°C water bath for exactly 2 min.

7. Add 200 μl LB.

8. Incubate at 37°C for 1 h without shaking.

9. Place 200 μl on LB-agar-ampicillin plates and use sterile glass-tool to distribute evenly on plate (if desired varying amounts of transformation reaction mix can be added to different plates).

10. Place plates in 37°C incubator overnight, transformant colonies should be visible in 12 to 16 h.

Characterization of subclones

Plasmid DNA can be isolation after overnight culture in 10 ml LB as described above. Restriction enzyme analysis comparing undigested, linearized and double digested plasmid DNA can be performed.

1.2 - *Isolation of Plasmid DNA*

Small-scale preparation of plasmid DNA

Small scale Isolation of plasmid DNA are performed using plasmid purification kit (Sigma Chemical Company, St. Louis, MO, USA). The plasmid DNA is resuspended in water.

Large-scale preparation of plasmid DNA

Large-scale preparation are done using CsCl ultracentrifugation procedures described by Maniatis et al. (Molecular Cloning, Cold Spring Harbor Laboratory, 1982), except that two 36 h CsCl spins are performed instead of only one. The yield of plasmid DNA when starting with 500 ml LB culture is between 100 and 400 μg.

1.3 - *Linearization of Plasmid DNA*

To allow the preparation of a CCK riboprobe the pBluescript plasmid is linearized with the restriction enzyme that allows for the RNApolymerase to 'fall off' after transcribing the riboprobe and reanneal to the polymerase binding site upstream of the CCK fragment. With high yields of plasmid DNA available from the large-scale preparation 20 μg DNA was digested at a time.

Linearization of pBluescript/CCK with HindIII.

1. Mix digestion reaction in a microcentrifuge tube
   20 μg (82 μl) plasmid DNA
   2 μl restriction enzyme (HindIII 10 units/μl)
   10 μl 10 x buffer
   6 μl H_2O.

2. Incubate for 1.5 h at 37°C in a waterbath.

3. Add equal volume phenol:chloroform:isoamyloalcohol (25:24:1, vol:vol:vol), vortex for 1 min, centrifuge 1 min at 12,000 g, transfer aqueous phase into new microcentrifuge tube.

4. Add equal volume chloroform:isoamyloalcohol (24:1, vol:vol), vortex, centrifuge and transfer aqueous phase as above.

5. Add 1/10 vol 3 M sodium acetate.

6. Add 2 vol ice cold 95% ethanol.

7. Incubate overnight at -20°C.

8. Centrifuge at 4°C, 12,000 g for 30 min.

9. Discard supernatant.

10. Wash pellet with 70% ethanol, centrifuge, and discard ethanol carefully.

11. Air dry pellet.

12. Resuspend pellet in 16 μl MQ H_2O.

13. Dialyze approx. 30 min on Millipore USWP filter against H_2O to achieve a final volume of 20 μl.

14. Quantify by UV absorption at 260 nm (e.g. 3 μl in 500 μl H_2O) and adjust volume to obtain final concentration of 1μg/μl.

15 Aliquot 1μl into microcentrifuge tubes and keep frozen at -20°C.

Appendix 2 - Isolation of RNA

2.1 - *Total RNA Isolation by Guadinine Thiocyanate/CsCl Procedure*

Large scale RNA isolation in swinging bucket rotor (Beckman SW 41 Ti rotor)
The large scale RNA isolation (1 g of tissue) was performed as according to Glimm (1991, Role of Growth Hormone in Mammary Biology. Ph.D. Thesis. University of Alberta. Appendix 2: p160-169) where solutions, methods and equipment are described in detail.

Mini GIT/CsCl RNA isolation in swinging bucket rotor (Beckman SW 50.1 rotor)
Refer to Glimm (1991) appendix 2 for handling and cleaning of equipment procedures. Solutions for RNA isolation are the same as for the large scale RNA isolation, briefly: Tissue amount of 20 to 120 mg can be used.

CsCl Solution:
5.7 M CsCl (95.97 g/ 100 ml = density 1.7 g/ml)
0.1 M EDTA (3.7 g $Na_2EDTAx2H_2O$/100 ml)
pH 7.0

GIT Solution
4 M guanidine isothiocyanate (47.28 g GIT/100 ml)
0.5% Na-lauryl-sarcosine (0.5 g/100 ml)
25 mM Tris-HCl, pH 7.5 (2.5 ml 1 M Tris-HCl stock/100 ml)
0.1 M ß-mercaptoethanol (700 μl 14.3 M stock/100ml)

1. Treat thin-walled polyallomer tubes (for SW 50.1 rotor; capacity 5 ml) with 0.1 N NaOH and rinse several times with RN-ase free H_2O.

2. Transfer 1.3 ml CsCl solution to each tube, cover with foil until use.

3. Add 3.6 ml GIT solution to each of 6 (15 ml) sterile polyallomer tubes.

4. Quickly add frozen tissue to GIT solution and then immediately homogenize at full speed for 60 seconds at room temperature with 5 mm polytron probe.

5. Centrifuge at 5,000g (in Beckman centrifuge, J2-21 rotor head) for 5 min at 4°C to remove cell debris.

6. Carefully remove homogenate from tube and layer onto the 1.3 ml CsCl cushion in the polyallomer tube.

7. Balance tubes by topping up each with GIT solution to approximately 1 mm from top.

8. Place tubes into buckets and screw on caps - not too tight!
9. Mount buckets on rotor and place rotor in ultracentrifuge Start ultracentrifuge and run at 42,000 rpm for 12 h at 20°C. (Due to the density being higher than 1.0 the speed has to be reduced from 50,000 to 42,000 rpm!)

10. After 12 h run, carefully remove tubes from buckets, aspirate carefully using a baked Pasteur pipette and 1 ml Pipetman without disturbing the clear pellet.

11. Wash pellet with 70% ethanol, remove ethanol carefully, let pellet air dry.

12. Resuspend in SET buffer or H_2O, if necessary vortex, freeze-thaw, and or waterbath heating at 65°C.

13. Store RNA solution at -70°C until quantification by UV absorption at 260 nm.

Mini GIT/CsCl RNA isolation in (Beckman) airfuge
Solutions and handling procedures are as described above, the airfuge method is modified from Rappolee et al. (1989, J. Cell. Biochem. 39:1-11). The method can be used for intestinal biopsy, consisting of mostly mucosa tissue, which is easily homogenized using a plastic pestle instead of a polytron probe.

1. Treat polyallomer tubes with 0.1 N NaOH and rinse well with RN-ase free water.

2. Transfer 100 μl of CsCl solution into each of 6 tubes.

3. Transfer 80μl GIT solution into a microcentrifuge tube.

4. One biopsy is homogenized in 80 μl GIT solution for 30 sec at high speed (800 rpm) in a microcentrifuge tube using plastic pestles (Kontes, Vineland, NJ, USA) attached to an upright drill.

5. Centrifuge for 30 sec at 5,000 g to bring all solids to the bottom of the microcentrifuge tube

6. Repeat homogenization, centrifuge, and homogenize again.

7. Centrifuge for 5 min at 5,000 g.

8. Transfer 75 μl onto the 100 μl CsCl cushion (Maximum fill in the tubes is 175 μl).

9. Carefully transfer tubes into the airfuge rotor, close plastic cover on rotor.

10. The sample density for 100 μl CsCl solution and 75 μl GIT solution is approximately

1.49 g/ml, so that the maximum speed has to be reduced from 110,000 rpm to 80,000 rpm. The run therefore has to be at 17.5 psi instead of 25 psi!

11. Centrifuge at room temperature for 1 h at 80,000 rpm (the minimum duration of the spin to be equivalent to the large scale 24 h spin in the SW 41 Ti rotor was calculated to be 0.45 h).

12. After the run is completed, carefully remove the GIT and CsCl solution, wash pellet with ethanol, airdry pellet, and resuspend RNA in SET buffer or H_2O.

13. Quantification the UV absorption can be achieved using quartz capillaries (Pharmacia, Canada) with volumes as low as 5 μl (e.g. 1 μl sample in 4 μl H_2O) to avoid using too much sample.

2.2 - *Total RNA Isolation by Trizol Procedure*

The RNA isolation using the Trizol reagent (Gibco BRL, Cat. No 155 96-026) was slightly modified from the company recommendations to fit the small scale required with the intestinal biopsy samples.

1. Add 50 μl of Trizol solution to one to two frozen intestinal biopsies into a microcentrifuge tubes

2. Homogenize for 30 sec at high speed (800 rpm) in a microcentrifuge tube using plastic pestles (Kontes, Vineland, NJ, USA) attached to an upright drill.

3. Centrifuge for 30 sec at 5,000 g to bring all solids to the bottom of the microcentrifuge tube.

4. Add 50 μl of Trizol solution and repeat homogenization and centrifugation.

5. Add 400 μl of Trizol solution and repeat homogenization.

6. Place on ice until all samples are homogenized.

7. Incubate samples at room temperature for 5 min.

8. Add 0.1 ml chloroform.

9. Shake tubes vigorously by hand for 15 sec.

10. Incubate the samples at room temperature for 5 min.

11. Centrifuge samples at 12,000 g for 15 min at 4°C.

12. Transfer aqueous tube into fresh microcentrifuge tube.

13. Add 0.25 ml of isopropanol, vortex tube and let precipitate overnight at -20°C (Gibco Trizol protocol recommends 1 h precipitation without followed phenol chloroform extraction, thus if desired continue from step 15 to step 23)

14. Centrifuge samples at 12,000 g for 15 min at 4°C.

15. Remove supernatant.

16. Airdry pellet.

17. Resuspend in 0.1 ml TE buffer.

18. Extract with equal volumes of phenol:chloroform:isoamyloalcohol (25:24:1, vol:vol:vol) and chloroform:isoamyloalcohol (24:1, vol:vol).

19. Add 1/10 volume 3 M sodium acetate, and 2.5 volume ice cold 95% ethanol.

20. Precipitate at -20°C overnight

21. Centrifuge samples at 12,000 g for 15 min at 4°C.

22. Remove supernatant.

23. Wash pellet with 70% ethanol and airdry pellet.

24. Resuspend in TE buffer or H_20, quantify by UV absorption, and store as described above.

2.3 - *Isolation of Polyadenylated RNA*

The isolation of polyadenylated RNA were modified slightly from Glimm, D.R. 1991. Role of Growth Hormone in Mammary Biology. Ph.D. Thesis. University of Alberta. Appendix 3: p170-173, and Maniatis et al. (Molecular Cloning, Cold Spring Harbor Laboratory, 1982). Solutions, methods, material and equipment are described in detail in Glimm (1991).

Solutions briefly:
Elution Buffer (EB):
10 mM Tris-HCl, pH 7.5
2 mM EDTA
0.1% SDS (added after autoclaving)

Binding Buffer (BB):
0.5 M LiCl
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1% SDS (added after autoclaving)

Wash Buffer (WB):
0.15 M LiCl
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1% SDS (added after autoclaving)

LiCl Solution:
10 m LiCl filtered with 0.45 μm Nalgene vacuum filter.

The procedure is a combination of the Maniatis batch affinity chromatography on oligo d(T) cellulose and the double column chromatography with oligo d(T) cellulose described by Glimm (1991). The requirements for oligo d(T) cellulose per mg total RNA vary from 0.6 g oligo d(T) cellulose /mg RNA (Maniatis) to 0.03 g oligo d(T) cellulose/mg RNA (Glimm). This protocol uses 0.06 oligo d(T) cellulose /mg RNA for the first column and continues with 0.01 g oligo d(T) cellulose /mg RNA for the second column as described in Glimm (1991). After suspension in buffer 0.25 g dry oligo d(T) yields approximately 1.0 ml swollen oligo d(T).

1. Total RNA is isolated by either large scale GIT/CsCl or large scale Trizol procedure. To isolate sufficient poly A^+ RNA for several RNA electrophoresis gels approximately 2 to 5 mg total RNA should be available. Poly A^+ RNA is approximately 1-2% of total RNA.

Column preparation

2. Weigh approximate amount of dry oligo d(T) in RN-ase free dish (0.06 g/mg RNA for batch affinity chromatography and 0.01 g/mg RNA for second column.

3. Transfer oligo d(T) into 5 or 15 ml sterile polypropylene tube.

3a. If previously used oligo d(T) is available the first wash is with 0.1 N NaOH/5 mM EDTA. Following this treatment wash with sterile MQ H_2O until the effluent is less than pH 8.0. Then continue to step 9.

4. Add 2 ml of EB for every 0.1 g dry oligo d(T) and gently invert tube several times to suspend oligo d(T).

5. Allow oligo d(T) to settle briefly (1-2 min) and aspirate the top layer of EB

containing the 'fine' particles of oligo d(T).

6. Repeat step 4 and 5 three more times.

7. The oligo d(T) for the **second** column is poured as a slurry in EB into the autoclaved column. Keep another column available to load the oligo d(T) from the batch affinity chromatography into, after the binding of the RNA to the oligo d(T) is completed.

8. Wash the oligo d(T) for the **second** column in the column with 10 column volumes of BB.

9. Wash oligo d(T) for batch chromatography with 10 volumes of BB, centrifuge at 1500 g for 4 min at room temperature and remove the supernatant.

10. Repeat step #9.

Binding of RNA to oligo d(T)

1. Determine exact volume of RNA to be applied to column.

2. Heat RNA solution at 65 °C for 10 min in water bath to denature RNA.

3. Add 1/20 volume 10 M LiCl to make RNA solution 0.5 M LiCl.

4. Gently apply RNA to tube with oligo d(T), seal tube.

5. Agitate gently for 15 min, centrifuge for 4 min at 1500 g at room temperature.

6. Remove but save supernatant at each step (the saved supernatants can be used to precipitate poly A^- RNA, which can be used as negative control in the gel electrophoresis)

7. Wash three times with 5 ml BB, centrifuge and remove supernatant each time.

8. Wash with 5 ml WB, centrifuge and remove supernatant.

9. Pour oligo d(T) as slurry into empty column.

10. Let drip until column stops, collect eluate. If some oligo d(T) remains along the wall of the column WB can be used to wash it to the bottom of the column. Determine the volume of oligo d(T) in the column.

11. Add two column volumes of EB. Collect eluate into a 5 ml tube. Determine exact recovery volume! (The eluate contains approximately 50% mRNA and 50% other RNA, thus a second column purification step is added).

12. Heat RNA at 65°C for 10 min in water bath to denature RNA.

13. Add 1/20 volume 10 M LiCl to make RNA solution 0.5 M LiCl.

14. Gently apply RNA to **second** column and collect eluate until column stops dripping.

15. Wash with two column volumes BB, thereby rinsing RNA that could be attached to the side of the walls to the bottom of column. Let drip and collect eluate.

16. Repeat step 15. two more times, collecting eluate.

17. Wash with 1.5 volumes WB, discard eluate.

18. Eluate poly A+ RNA with two volumes EB (if desired the eluate can be collected in fractions of e.g. 1 ml to later determine the ones containing the most RNA).

19. To the final eluate add 1/10 volume 3M sodium acetate and 2.5 volumes cold 95% ethanol.

20. Incubate overnight at -20°C.

21. Centrifuge 30 min at 4°C at 12,000 g, aspirate supernatant and air dry pellet.

22. Resuspend in TE or SET buffer or H_2O.

22. Quantify by UV absorption at 260 nm, aliquot in convenient amounts (e.g. 5, 10, 15, and 20 μg) and store at -70°C.

Appendix 3 - Northern Blot Analysis

The procedures from Glimm (1991 Ph.D. Thesis, University of Alberta) were followed.

3.1 - RNA Gel Electrophoresis and Northern Transfer

For procedure description, solutions, and equipment see Glimm (1991. Ph.D. Thesis, University of Alberta, 1992, Appendix 4: p174-178.

Modified are only the following procedures:

A larger gel electrophoresis unit required the use of larger gels:

1. Into 250 ml Erlenmeyer flask add 1.5 g agarose.
2. Add 130 ml MQ H_2O
3. Microwave 1-2 minutes to boil, interrupt several to swirl to aid dissolving agarose.
4. Add 10 ml 10 x MOPS, swirl flask to mix.
5. Take out small amount of hot gel solution to seal gel unit.
6. Allow gel to cool to 50 to 60°C, then add 8.1 ml 37% formaldehyde and mix gently to avoid bubbles.
7. Immediately pour gel solution into gel unit with comb in place. Remove bubbles with Pasteur pipette.
8. Allow to stand for 1 h before use.

Larger number of samples to be run on one gel required the use of combs with 20 or 24 wells. These wells hold only 15 or 10 μl loading dye and sample mix, instead of 30 μl (16 well combs).
RNA samples were therefore placed into a cleaned speed vac centrifuge until just dried (avoiding overdrying, because this causes difficulties while resuspending) and resuspended into the loading dye, instead of mixing 6 μl sample solution with 25 μl loading dye as described by Glimm, 1992.

After completed transfer a photograph was taken of the Northern transfer membrane. The Ethidium bromide stained 28S rRNA can be used to correct for loading differences between different lanes

3.2 - Probe Labeling and Northern Hybridization

For procedure description, solutions, and equipment see Glimm (1991. Ph.D. Thesis, University of Alberta. Appendix 5: p179-186).

Modified are only the following procedures:

During the **antisense riboprobe preparation** the T3 RNApolymerase is used, which requires incubation at 37°C for transcription instead of 40°C.

To calculate the **ratio of incorporated to un-incorporated label:**

1. Remove 2 μl of reaction mix before the cDNA template is digested.

2. Dilute the 2 μl of reaction mix into 498 μl of TE buffer.

3. To determine the incorporated radioactivity, a 5 μl aliquot of the dilution is spotted on a glass fiber filter disk, washed three times with 50 ml of ice cold 10 % (wt:vol) TCA containing 1% (wt:vol) sodium pyrophosphate, once with 95% ethanol at room temperature. The filter is then dried.

4. For confirmation of total radioactivity a 5 μl aliquot of the dilution is spotted on a glass fiber filter disk and dried.

5. The radioactivity on both filter disks is counted by liquid scintillation counting.

6. The incorporated radioactivity is 2750 x cpm counted of filter disk from step 3.
   The total radioactivity is 2750 x cpm counted of the filter disk from step 4.

The ratio incorporated to un-incorporated radioactivity in our labeling reactions are normally 60 to 70%.

The **prehybridization and hybridization of the membranes** are performed in hybridization tubes in a rotation hybridization incubator, instead of plastic bags in a waterbath. The volumes of prehybridization and hybridization solution are kept constant at 0.15-0.25 ml solution/cm^2 of membrane as described by Glimm (1991).

The membranes that were hybridized with the CCK riboprobe are washed as described by Glimm (1991) with the **most stringent wash** being performed at 75°C for 35 min to reduce the amount of non-specific signals of rRNA with the CCK riboprobe. For the membranes that are hybridized with the proglucagon riboprobe the most stringent is performed at 70°C for 15 min. Autoradiogram exposure ranges from 8h for the proglucagon signal to 24 h for the CCK signals.

3.3 - Quantitation by Densitometry and Interassay Standardization

Following the development of the films, the signals are analyzed using an imaging densitometer (BioRad Laboratories, Mississauga, ON) and expressed in units of adjusted volume (OD x mm^2).

The photograph of Ethidium bromide stained 28S rRNA on the Northern transfer membrane is also analyzed for the adjusted volume.

The correction for varying amounts of RNA loaded between different lanes is achieved by dividing the adjusted volume for the specific signal by the adjusted volume for the 28S rRNA from the transfer membrane.

The completion and analysis of a 'dosage test' is recommended before using samples collected during experiments to determine:
- the optimal amount of total RNA or poly A^+ RNA required to detect a specific signal,
- different transcript sizes to determine agreement with sizes reported for the specific RNA transcript size in the literature,
- the amount of non-specific binding of the probe with ribosomal RNA, which will be visible in poly A^- RNA, but not in poly A^+ RNA.

If more samples require analysis than can be separated on the same electrophoresis gel an internal standard sample (e.g. positive and negative control) should be included into every gel. This will allow for the interassay variation coefficient to be calculated from standard deviation of the internal standard samples between assays divided by the mean value, which is an indication for the repeatability of the assay. The interassay variation coefficient for the CCK-hybridized membranes has been 4% and for the proglucagon hybridized membranes has been 1.2%.

A comparison of the RNA isolation using Mini GIT-CsCl and Trizol procedure was performed on several duodenal biopsies of different sizes. The samples were analyzed by Northern blot analysis and on figure A3.1 it can be seen that the resulting CCK mRNA signal was very similar for either method.

Appendix 4 - Ribonuclease Protection Assay (RPA)

The procedure for the ribonuclease protection assay of Gilman (1987, Ribonuclease protection assay. In Ausubel FM (ed) Current Protocols in Molecular Biology. John Wiley & Sons, New York) was followed and slightly modified.

Solutions briefly:

5 x PIPES:
220 mM PIPES,
2M NaCl,
5 mM EDTA, pH 6.4.

to make 100 ml:
6.05 g PIPES, di-sodium salt
11.69 g NaCl
0.186 g EDTA,
adjusted pH to 6.4. Can be frozen at -20°C.

Hybridization solution is 1 part 5 x PIPES and 4 part de-ionized formamide (vol:vol).

Ribonuclease digestion solution:
10 mM Tris-HCl, pH 7.5
300 mM NaCl
5 mM EDTA

to make 100 ml:
0.158 g Trisma-HCl,
1.75 g NaCl
0.186 g EDTA.2Na

RNA Loading buffer:
80% formamide
1 mM EDTA, pH 8.0
0.1% bromophenol blue
0.1 % xylene cyanol

to make 10 ml:
8 ml formamide
20μl 0.5 M EDTA pH 8.0
10 mg bromophenol blue
10 mg xylene cyanol
1.6 ml MQ H_2O,
can be frozen at -20°C.

RN-ase solutions:

RN-ase A stock = 5 mg/ml, can be frozen at -20°C
working solution = 40 μg/ml

T1 RN-ase (Sigma) stock = 1.2μg/μl, can be frozen at -20°C
working solution is 2 μg/ml.

to make 10 ml RN-ase digestion solution:

80 μl stock RN-ase A
16.5 μl stock RN-ase T1

in 10 ml ribonuclease digestion solutions.

Antisense riboprobe preparation:
The same procedure is used as described by Glimm (1991), appendix 5.

The following modifications are necessary:

The complete digestion of the cDNA template is absolutely essential to allow binding of the riboprobe to the RNA. If necessary the amount of DN-ase can be increased .

The Sephadex G-50 spin column is equilibrated with 200 μl 1 x PIPES instead of 100 μl TE buffer.

To decrease the binding of smaller transcription fragments with sample RNA the riboprobe can be gel-purified. This will also remove cDNA template that may not be completely digested. After overnight precipitation of the probe (spin column is not necessary if gel separation is performed) and resuspension of the probe in loading buffer, the RNA fragments are separated on a small polyacrylamide/urea gel. The upper glass plate of the gel unit is removed and in the darkroom the gel is exposed for approximately 1 min to an x-ray film, which is immediately developed. The x-ray film is aligned with the gel, so that the band containing the riboprobe can be excised. The probe is eluted out of the gel slice by incubation for 2 to 4 h in an equal volume of 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS and is subsequently precipitated, and can be added to the hybridization buffer.

Preparation of hybridization buffer:
Formamide is deionized as described by Glimm (1991), appendix 5.

1. Add 1 part 5 x PIPES to 4 part of deionized formamide (vol:vol).

2. Mix sufficient riboprobe and hybridization solution to provide 2 to 5 x 10^6 dpm in 30 μl solution.

Preparation of samples and solution hybridization:

1. Aliquots of the desired samples are precipitated with 1/10 vol 3 M sodium acetate and 2.5 vol 95% ethanol, overnight at -20°C. Include 15 μg tRNA as a negative control along with the RNA samples. Different amounts of sample RNA (e.g. 10, 15, 20, 30 μg) should be included to determine linearity of signal detection. As a positive control add a small amount of the probe alone, which is not digested! with RN-ase digestion buffer.

2. Microcentrifuge samples for 30 min at 4°C at 12,000 g, remove supernatant and let pellet airdry.

3. Add 30 μl of hybridization buffer containing the riboprobe to the sample.
(Do the subsequent procedures behind a Plexiglas screen due to P^{32} radioactivity)

4. Incubate for 5 min at 85°C to denature sample, vortex several times during the 5 min.

5. Place in a 45 °C water bath for overnight solution hybridization.

Ribonuclease digestion:

The use of a separate Pipetman and (or) aerosol pipette tips to avoid contamination of the Pipetman with ribonucleases is recommended to avoid future RNA degradation.

1. To each hybridization reaction add 350 μl digestion buffer containing RN-ases A and T1. Incubate for 45 min at 30°C.

2. Add 20 μl of 10% SDS and 2.5 μl of proteinase K (20 mg/ml)
Incubate for 15-20 min at 37°C.

3. Extract once with 400 μl phenol:chloroform:isoamyloalcohol (25:24:1, vol:vol:vol), removing exactly 360 μl of the aqueous phase into a new microcentrifuge tube.

4. Extract once with 360 μl chloroform:isoamyloalcohol (24:1, vol:vol), removing exactly 320 μl of the aqueous phase into a new microcentrifuge tube.

5. Add 2 μl of 10 mg/ml t-RNA solution to precipitate the RNA/probe fragments!

6. Add 1/10 vol 3 M sodium acetate and 2.5 vol 95% ethanol, and precipitate at -20°C for 30 min.

7. Centrifuge sample for 25 min at 4°C at 12,000 g.

8. Discard supernatant, airdry pellet thoroughly, because any remaining ethanol will cause the sample to float out of the well after loading into polyacrylamide gel!

9. Add 3 μl of loading buffer and resuspend thoroughly for 5 min (if necessary longer) at 85°C with vortexing. Then place sample on ice.

10. Analyze on a 6% polyacrylamide/7M urea gel
(Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory).

11. Following electrophoresis, place the non-silanized 'rabbit ear' plate on a flat surface. Separate the glass plates, and fix gel by soaking the non-silanized plate with the gel in 10% methanol, 10% acetate in water for 15 min.

12. Drain for 15 min and absorb the gel to a 3M filter paper of the same size as the gel.

13. Seal gel and filter paper into a plastic bag and expose to a Kodak XAR X-(OMAT) AR 8" x 10" x-ray film with intensifying screen. Audioradiographic exposures ranged from 2 to 14 days.

Appendix 5 - Cloning of Bovine CCK cDNA using Polymerase Chain Reaction (PCR)

In order to use a species specific riboprobe during the ribonuclease protection assay to increase its sensitivity, the cloning of the bovine CCK cDNA was prepared.

5.1 - Design of Primers

Two sets of primers to allow nested amplification were designed, the first set based on the rat DNA sequence, and the second based on the porcine DNA sequence within a region of nearly complete homology between the species human, rat, monkey, mouse, and pig.

The following guidelines for designing oligonucleotide primers were followed (Rapollee, Amplifications, A forum for PCR users, p 5-7):

- Oligonucleotide primers were 22 mers with a Tm of 68 and 70 °C (near 72°C) to allow PCR annealing plateau at the optimal stringency.
- The framed sequence was 610 and 520 base pairs, each set with only one main amplification product predicted when checked with the program 'amplify'.
- Primers covered areas of inter-species homology for the gene in question, and were in the translated region to increase chances of species homology.
- Primers were designed to avoid primer dimers or secondary structures as detected by the programs 'gene jockey' and 'amplify'.
- Primers had a balanced G/C and A/T concentrations.
- Primers were specific for the CCK gene and did not report any other matches when checked with Sequence data banks.
- Primers were designed to have TT nucleotide at 3' end, which will work better at mismatches.

The primers were diluted to desired concentration of 50 μM with water and aliquots stored at -20°C, to avoid contamination of the stock solutions.

5.2 - Optimizing Reverse Transcriptase (RT) and Polymerase Chain Reaction (PCR)

Primers used:

DGL 18 = CCK 1.1s: designed according to rat CCK cDNA

A = 5, C = 6, G = 6, T = 5, X = 0; 22 bases;

T_m= 4 °C (G+C) + 2 °C (A+T) = 68 C,

L_n= 2 * (G+C)+ (A+T) = 34

$T_p = 22 + 1.46 * (L_n) = 71.6$ °C

Sequence: 5' > ACTTAGCTGGACAGCAGCCGTT <3'

DGL 19 = CCK 2.14: designed according to rat CCK cDNA

A = 8, C = 5, G = 7, T = 2, X = 0; 22 bases;

$T_m = 4$ °C (G+C) + 2 °C (A+T) = 68 C,

$L_n = 2 * (G+C) + (A+T) = 34$

$T_p = 22 + 1.46 * (L_n) = 71.6$ °C

Sequence: 5' > GGAGCCACCAGAGGGAAACATT <3'

DGL 20 = sense 1: designed according to pig CCK cDNA within species-homologous region

A = 3, C = 5, G = 8, T = 6, X = 0; 22 bases;

$T_m = 4$ °C (G+C) + 2 °C (A+T) = 70 C,

$L_n = 2 * (G+C) + (A+T) = 35$

$T_p = 22 + 1.46 * (L_n) = 73.1$ °C

Sequence: 5' > ATGAACGGCGGCTTGTGCCTGT <3'

DGL 21 = antisense 11: designed according to pig CCK cDNA within species-homologous region

A = 3, C = 3, G = 10, T = 6, X = 0; 22 bases;

$T_m = 4$ °C (G+C) + 2 °C (A+T) = 70 C,

$L_n = 2 * (G+C) + (A+T) = 35$

$T_p = 22 + 1.46 * (L_n) = 73.1$ °C

Sequence: 5' > GAGGTGCGTGGTTGCATTGGAC <3'

Primers sense.1 and antisense.11 are nested within CCK1.1s and CCK 2.14

PCR-fragments predicted:

CCK 1.1s	& CCK 2.14	610 bp fragment
CCK 1.1s	& antisense.11	577 bp fragment
sense.1	& CCK 2.14	553 bp fragment
sense.1	& antisense.11	520 bp fragment

Conditions

The RT reaction is performed using different amounts (0.5 to 3 μg) of total RNA isolated from biopsy tissues, where CCK mRNA is known to be sufficient high to detect by Northern analysis. The RT can also be performed using 0.1 to 1 μg of poly A+ RNA, however, it has been reported that the LiCl buffer used during the isolation of poly A+ RNA can interfere with the reverse transcriptase enzyme.

RT mix:

2 μl dNTP mix (1 mM each)
2 μl 10 x PCR buffer
1 μl RNA
0.21μl antisense primer (75 ng)
13 μl H_2O

RNA is denatured for 5 min at 65°C, then RN-ase inhibitor and reverse transcriptase added.

0.5 μl RN-ase inhibitor (20 units added after denaturation)
2 μl MM LRT (200 units)

The RT reaction is incubated for 42°C for 1 h, and reaction stopped by boiling at 95 to 100°C for 10 min.
1, 5, or 10 μl of RT is included into the PCR and the amount of dNTP and antisense primer carry over calculated and compensated for.

The PCR conditions are designed using a 'hot start' procedure, which avoids the annealing of primers to template at less stringent temperatures during the heating of the PCR prior to reaching the annealing temperature. This is achieved by separating the

bottom layer (containing the primers) from the upper layer (containing the template) by melting a wax pellet that covers the lower layer and solidifies before the upper layer is added. After a temperature of approximately 75° is reached the wax layer melts and floats to the top, which enables the template and primers to anneal.

Lower Mix:
1.25 µl 10 x PCR buffer
1 - 10 µl template from RT mix.
0.56 µl antisense primer (200 ng, needs to be adjusted for carry over from RT)
0.56 µl sense primer
0.64-9.14 µl H_2O

Mix into PCR tube, add wax pellet, melt at 75°C, and let solidify on ice.

Upper Mix:
5 µl 10 x PCR buffer
2-3 µl 50 mM $MgCl_2$ (needs to be optimized for 2 or 3 mM)
2.4 µl 10 mM dNTP mix (needs to be adjusted for carry over from RT)
1 µl Taq Polymerase (2.5 units)
26-27 µl H_2O

The PCR temperature is chosen to have four cycles of very stringent annealing temperature, which is followed by twenty nine cycles of more moderate stringent annealing conditions. This strategy allows for the specific matches of primer and template which can anneal under the very stringent conditions to get an increased amplification before the less perfect matches can anneal under the moderately stringent conditions.

The specific PCR - conditions are:

Start denaturing	95°C for 3 min
Cycle 1 to 4:	
denaturing	95°C for 45 sec
annealing	70°C for 1 min
elongation	72°C for 1 min
Cycle 5 to 29:	
denaturing	94°C for 1 sec
annealing	65°C for 1 min
elongation	72°C for 1 min
end elongation	72°C for 7 min

Separate PCR amplified products in agarose gel (add Ethidium Bromide to visualize DNA fragments). To avoid contamination of PCR amplification products onto Pipetman

a separate set and (or) aerosol pipette tips should be used when working with amplified products or setting up PCR mix.
The wax layer on top of the reaction mix will have to be penetrated using a pipette tip.
Use 18 μl of the reaction mix and add 2μl loading dye, mix, briefly centrifuge and load onto gel. Use molecular weight markers of the similar size of the expected fragment.

If the appropriate fragment size is detected the band can be excised and the RNA eluted with e.g. the QIAGEN PCR purification kit (Qiagen inc., Chatsworth, CA, USA). The ends of the fragment are polished using the Klenow fragment to increase the efficiency for the blunt end ligation described in the PCR-Script cloning kit (Stratagene, La Jolla, CA).
After successfully cloning the transformants can be screened by restriction enzyme digest to confirm the insert and by southern hybridization to confirm the specificity of the clones before having the sequencing done.

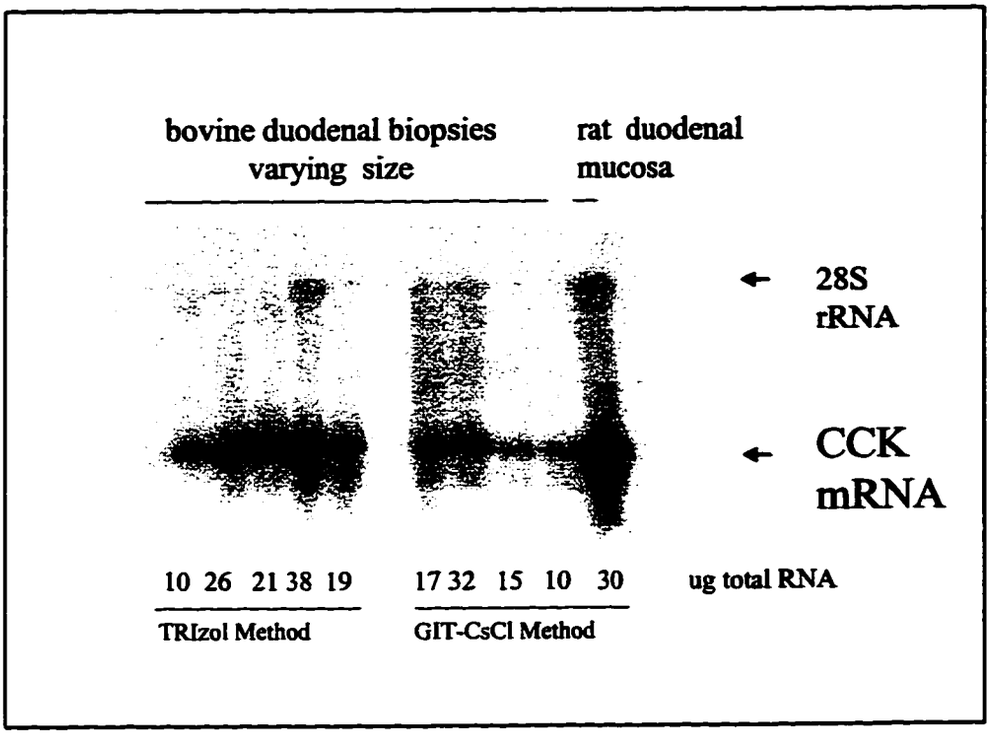

FIGURE A3.1 - CCK mRNA Abundance in Duodenal Biopsies

Autoradiogram depicting the CCK mRNA abundance of duodenal biopsy samples of varying size. The location of 28S rRNA and CCK mRNA are indicated. The two RNA isolation methods using Trizol or GIT-CsCl give comparable results detecting a CCK mRNA signal in10 to 38 μg total RNA.