EFFECTS OF β-CASOMORPHINS ON METABOLISM OF DAIRY COWS

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SUMMARY

1. The studies presented in this thesis investigated some metabolic actions of β-casomorphins in dairy cows. Three β-casomorphins (β-casomorphin-4-amide, -5 and -7) were used. Investigations of the actions of the β-casomorphins in dairy cows require gram quantities of each peptide. This was achieved by synthesising the peptides using solid phase peptide synthesis combined with FMOC chemistry.
2. The first experiment examined possible stimulatory actions of the peptides on amino acid uptake by the mammary gland using explants of lactating rat mammary glands as a model *in vitro* system. There was no evidence of their actions on amino acid uptake by the mammary gland as judged by the lack of their effects on the uptakes of four amino acids (glutamate, histidine, leucine and lysine). Because of the inability to demonstrate effects of the peptides at the mammary gland level, it was decided to focus on their effects at the level of the gut.
3. As a first step, the extent of ruminal degradation of the β-casomorphins was determined in incubations in rumen liquor *in vitro*. All three peptides were degraded rapidly with half-lives of only 15 to 20 minutes. In a subsequent experiment, an attempt was made to chemically protect the peptides from the ruminal degradation using N-terminal acetylation. The half-lives of the N-acetyl β-casomorphins were markedly increased to $6\frac{1}{2}$, $7\frac{1}{2}$ and 4 hours for β-casomorphin-4-amide, -5 and -7 respectively. However, it was recognised that, whilst the method would be a simple and effective method for the protection of the peptides, further developments of the method would be necessary to ensure their release in active form at their sites of their action *in vivo*.

4. In experiments on the actions of the β-casomorphins *in vivo*, the peptides were infused direct into the abomasum. The first experiment compared hormonal responses to the abomasal infusion of sodium caseinate, a potential source of β-casomorphins, and an acid hydrolysate of casein, which was not a potential source of β-casomorphins. There were clear suggestions of differences in responses of some hormones, notably insulin and GIP. In the second experiment, effects of abomasal infusions of mixtures of the β-casomorphins themselves at three different dose levels on changes in concentrations of the hormones were investigated in lactating dairy cows. There was no clear effect of the β-casomorphin infusions on insulin concentrations except for some tendency towards inhibition at some time points. However, the incremental response of glucagon to the β-casomorphin infusions was linearly increased ($P < 0.05$) leading to statistically significant decreases in the insulin / glucagon ratio at 4 hours by all β-casomorphin infusions compared with control.
5. In the final section of the thesis, attention was focused on the possible inhibitory effects of the β-casomorphins on insulin secretion. Three experiments were carried out. Experiment 1 was designed to detect the inhibitory action of the β-casomorphins on the insulin level prestimulated by an abomasal infusion of glucose. The insulin concentration rise was significantly inhibited by the β-casomorphins ($P < 0.05$). The inhibitory action of β-casomorphin was shown to be compatible with the action of SS-28 as judged from the effects of SS-28 on the insulin secretion when administered intravenously in the same experiment. However, the inhibitory action was not evident in the next experiment in which lactating animals were used, probably because of differences in the sensitivity of insulin secretion in lactating versus non-lactating animals. The final experiment confirmed the insulinopenic effect of the β-casomorphins by demonstrating their inhibitory action on the insulin concentrations prestimulated by an intravenous infusion of glucose.

6. Taken together, the results of the experiments described show effects of β-casomorphin infusions on circulating levels of hormones in the ruminant. The most pronounced effect was the modulation of the insulin response to abomasal or intravenous administration of glucose.

DECLARATION

This thesis has been compiled by myself and has not been offered in any previous application for a degree. With the exceptions of 1) feed and milk analysis by Mrs. I. Stewart and Mr. J. Davidson, 2) GLP-1 RIA in Experiment 1 of Chapter 5 by Dr. P. Martin and 3) IGF-1 RIA in Experiment 1 of Chapter 5 by Mrs. M. Gardner, I declare that all the work contained in this thesis is my own, undertaken under the supervision and guidance of Drs D. G. Chamberlain, J. -J. Choung, D. B. Shennan and R. J. Wallace.

Tae-Gyu Kim

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I would like to take the opportunity to thank all Koreans at the Hannah who has kept me entertained in a traditional (?) way for many years despite the Scottish weather, which can, sometimes, be depressing!

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ACTH	Adrenocorticotropic hormone
ADF	Acid detergent fibre
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
bST	Bovine somatotrophin
CAH	Casein acid hydrolysate
cAMP	Adenosine 3', 5' cyclic monophosphate
CCK	Cholecystokinin
CSN	Casein
DAMME	D-Ala^2-MePhe^4-Met-enkephalin-(O)-ol
DNAse	Deoxyribonuclease
DP-IV	Dipeptidyl-peptidase IV
DPM	Disintegrations per minute
EDTA	Ethylene diaminetetra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FAA	Free amino acids
FMOC	9-fluorenylmethoxycarbonyl
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GIP	Glucose-dependent insulinotrophic polypeptide
GLIs	Glucagon-like immunoreactants
GLP-1	Glucagon-like peptide-1
GRP	Gastrin-releasing peptide
HSA	Heptane sulfonic acid
HPLC	High-performance liquid chromatography
IgA	Immunoglobulin A
IGFBPs	IGF-binding proteins
IGF-I	Insulin-like growth factor-I
IgG	Immunoglobulin G

IgM	Immunoglobulin M
LHRH	Luteinising hormone-releasing hormone
MALDI-TOF-MS	Matrix-assisted laser desorption/ionizationdistortion time-of-flight mass spectrometer
ME	Metabolisable energy
N	Nitrogen
NDF	Neutral detergent fibre
NGF	Nerve growth factor
PDV	Portal-drained viscera
PEG	Poly ethylene glycol
PP	Pancreatic polypeptide
PYY	Peptide YY
RIA	Radioimmunoassay
RNAses	Ribonucleases
RUP	Ruminally undegradable protein
SEM	Standard error of the means
SLI	Somatostatin-like immunoreactivity
SPI	Soya protein isolates
SRIF	Somatotrophin-release inhibiting factor
SS-14	Somatostatin-14
SS-28	Somatostatin-28
TBTU	2-[1H-Benzotriazol-1-yl]-1,1,3,3--tetramethyluronium tetrafluoroborate
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
VFA	Volatile fatty acids
VIP	Vasoactive intestinal peptide

CHAPTER ONE

REVIEW OF LITERATURE

1.1. INTRODUCTION

Infusions of casein or partial hydrolysates of cassein into the abomasum of the dairy cow elicit greater increases of milk production than do iinfusions of corresponding mixtures of free amino acids (Choung and Chamberlain, 1995a). Furthermore, it has been demonstrated that the magnitude of the increase in the yield of milk protein is directly proportional to the proportion of peptide-bound amino acids in the hydrolysates infused (Choung and Chamberlain, 1995b).

These results are compatible with an involvement of peptides derived from casein. Casein is a rich source of biologically active peptides, those produced from β-casein (the β-casomorphins) being the most intensively studied. These are reported to affect the physiology of the gut in a number of ways, ranging from alterations of intestinal motility to increases in the rate of absorption of amino acids and stimulation of the release of various gut hormones. However, there is very little information available on the effects of β-casomorphins (or other diet-derived bioactive peptides)) in the ruminant. An understanding of the role of diet-derived bioactive peptides in the ruminant in relation to their action via endocrine regulation may ultimately allow not only development of a powerful tool to manipulate milk composition and improve efficiency of utilisation of dietary nutrients for production in the ruminant but also more accurate evaluation of feed protein sources.

In Chapter 1 of this thesis, the literature relating to the digestion and the metabolism of protein in the ruminant and their regulation by major metabolic hormones and gut hormones is reviewed, as well as the possible involvement of biologically active peptides

from dietary protein, as an introduction to a series of investigations of the actions of biologically active peptides derived from casein in the metabolism of dairy cows.

1.2. PROTEIN DIGESTION IN THE RUMINANT

1.2.1. Characteristics of the digestive tract of the ruminant

The region within the digestive tract of the ruminant that corresponds to the gastric stomach of the simple-stomached animal is divided into four different compartments (figure 1-1). The first two compartments, the reticulum and rumen, which are functionally related and are separated only by a layer of stomach wall, therefore are often described as the reticulo-rumen. This is where the ruminant houses symbiotic microorganisms and where extensive fermentation of nutrients by ruminal microorganisms occurs. The positioning of an organ of extensive fermentation before the major site of absorption in the small intestine makes the ruminant digestive system unique. As a consequence of the rumen fermentation of dietary material, ATP is generated for microbial growth and other end products of fermentation are produced.

Microbes and dietary components that escape microbial attack flow through the omasum, where some electrolytes and water are removed, to the abomasum. The abomasum has the same function as the glandular stomach in monogastric species secreting hydrochloric acid and pepsin. From the abomasum, digesta flow into the duodenum, where bile and pancreatic enzymes break down bacteria and undegraded food residues to sugars, long-chain fatty acids and amino acids, prior to absorption. Undigested material passes from the small intestine to the caecum and colon, where further microbial fermentation and some absorption of volatile fatty acids (VFA) occur.

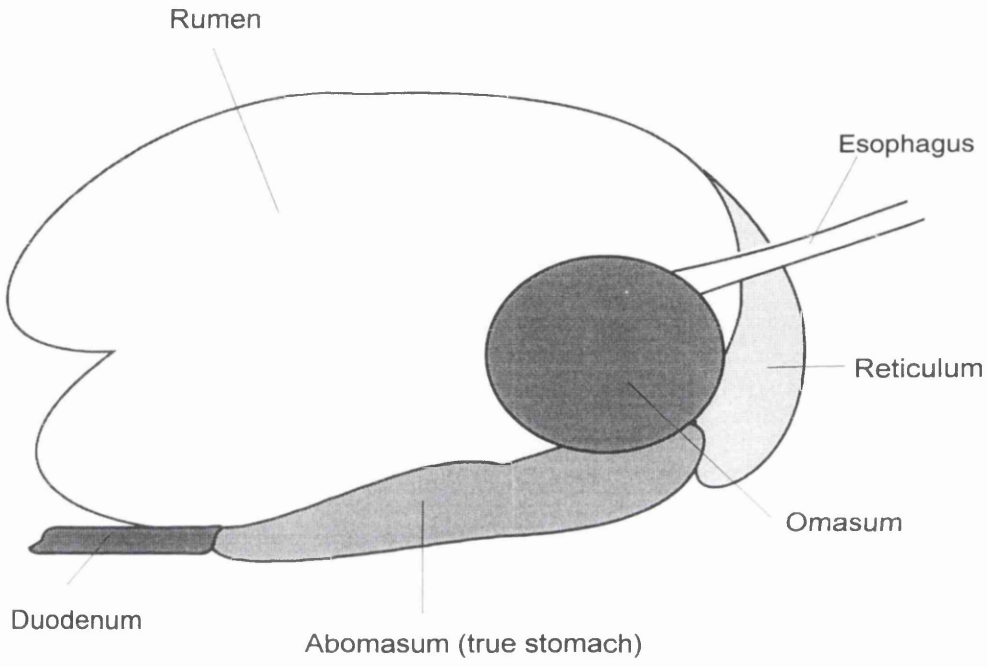


Figure 1-1. A diagram of the digestive tract of the ruminant (side view, right side).

1.2.2. Ruminal digestion of protein

Dietary protein ingested by ruminant animals is subjected to extensive degradation by ruminal microbes, both bacteria and protozoa, releasing oligopeptides and then small peptides and amino acids (Broderick *et al.*, 1991). Between 30 % and 50 % of the bacteria isolated from rumen fluid have proteolytic activity towards extracellular protein (Fulghum & Moore, 1963; Prins *et al.*, 1983) and bacteria classified as *Prevotella ruminicola* (formerly *Bacteroides ruminicola*) and *Butyrivibrio fibrisolvens* are commonly thought to play a major role in ruminal protein degradation (Wallace and Cotta, 1988; Wallace, 1994), with *P. ruminicola* isolates possessing an activity profile most similar to that of whole ruminal contents (Wallace and Brammall, 1985). Protozoa also play an important role in the degradation of particulate and microbial proteins (Hino and Russell, 1987; Ushida *et al.*, 1991), and produce a variety of enzymes with different specificities (Forsberg *et al.*, 1984; Newbold *et al.*, 1989).

Although the mixed ruminal microbiota has no absolute amino acids requirement (Virtanen, 1966), up to about half of microbial N can be derived from nonammonia N sources, which would be predominantly peptides and amino acids (Nolan, 1975; Leng and Nolan, 1984). Most reports of the uptake of ^{14}C-amino acids and peptides indicated that the mixed microbial population preferentially incorporated peptides rather than free amino acids (Wright, 1967; Prins *et al.*, 1979; Copper and Ling, 1985). However, more recent studies reported a preference for amino acids over peptides by some microbial species (Westlake and Mackie, 1990; Ling and Armstead, 1995), and mixed population (Armstead and Ling, 1993). It has been suggested that the numbers of *P. ruminicola* in the mixed population may decide the preference for nonammonia N sources incorporated into microbial proteins as *P. ruminicola* prefers peptides to amino acids, and this organism can comprise more than 60 % of the total microflora (Wallace, 1996).

Ruminal peptide breakdown is a two-stage process (figure 1-2). The great majority of peptidase activity in ruminal contents is similar in specificity to dipeptidyl aminopeptidase Type-I that cleaves dipeptides rather than single amino acids from N-terminal of peptide chains (Wallace and McKain, 1989; Wallace *et al.*, 1990; Wallace *et al.*, 1993b). The dipeptides released as a result of dipeptidyl aminopeptidase activity are then broken down by separate dipeptidases to free amino acids.

Individual amino acids may be incorporated into microbial protein or deaminated intracellularly to VFA, ammonia, CO_2 and CH_4. For many years, it had been assumed that deamination was carried out by a large number of the principal species of ruminal bacteria that had been shown to produce ammonia from protein or protein hydrolysates. However, more recent studies (Chen and Russell, 1988; Russell *et al.* 1988, Chen and Russell 1989, Russell *et al.*, 1991; Allison *et al.*, 1992; McSweeney *et al.*, 1993; Paster *et al.*, 1993) suggest that amino acid deamination is carried out by two distinct bacterial populations of either low activity with high numbers or high activity with low numbers (see Wallace, 1996).

The degradation of protein in the rumen is influenced by a number of factors, some of which are related to diet, others to the animal. The secondary and tertiary structure of proteins is an important determinant of degradability. Proteins with extensive crosslinking, such as disulphide bonds (e.g. albumins and immunoglobulins) appear to be more resistant to degradation (Nugent and Mangan, 1978). By the same principle, the use of controlled Maillard reactions between soybean meal and xylose (Cleale *et al.*, 1987) and pretreatment of leaf protein with formaldehyde, which causes methylene crosslinking, have both proven to be effective ways to reduce the rate of proteolysis (Ferguson *et al.*, 1967, Mangan *et al.*, 1980). More recently N-terminal modification of amino acids and peptides has also been shown to reduce their ruminal degradation rates (Wallace *et al.*, 1993a; Wallace, 1994).

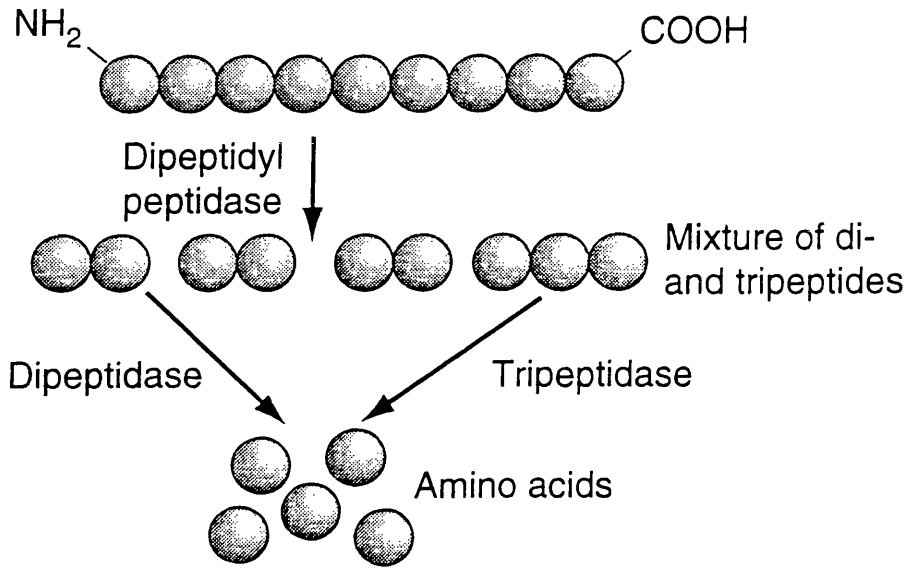


Figure 1-2. Biphasic breakdown of peptides by rumen microorganisms (After Wallace, 1996).

In general, soluble proteins are more rapidly and completely degradable in the rumen than those that are insoluble. Indeed, solubility of proteins does correlate well with degradability of proteins as long as it applies to a group of similar feeds (Beever *et al.*, 1976). However, protein solubility as a measure of protein degradation can lead to serious error when applied across a variety of feeds (Nugent and Mangan, 1978; Leng and Nolan, 1984).

The products of protein degradation, peptides, free amino acids, and ammonia, are utilised for protein synthesis by ruminal microbes. The microbes almost always improve the protein status of ruminants consuming low protein diets through protein synthesis from degraded feed protein and efficient use of urea recycled to the rumen via saliva and directly across the ruminal wall, such that more protein reaches the small intestine than was ingested. However, when high-protein diets are fed, the production of ammonia often exceeds the rate at which it is reassimilated into microbial protein, and the excess is absorbed and transported to the liver, then excreted primarily as urea. To minimise this 'waste' of protein, the objectives of research in this field have been twofold. One is to maximise microbial protein synthesis by optimising ruminal degradation of carbohydrates and proteins and the other is to minimise protein breakdown in the rumen, and thereby to increase the 'bypass' dietary protein (i.e. protein escaping degradation in the rumen) reaching the lower tract.

Microbial protein flowing to the small intestine accounts for 40 to 80 % of daily amino acid supply of ruminants and it has a relatively good amino acid balance (Clark *et al.*, 1992). The primary determinant of the amount of protein synthesised in the rumen is the energy available for microbial growth. The amount of energy required for the formation of microbial cells is variable and can be considerably above theoretical estimates (Hespell and Bryant, 1979). This is a reflection of modifying factors such as growth rate of rumen bacteria, turnover of microbial protein, supplies of nitrogen and other nutrients,

variation of the composition of organisms, and differences of metabolic pathways of microorganisms (Hespell and Bryant, 1979; Leng, 1981; Leng and Nolan, 1984). Minerals, especially sulphur and phosphorus, also can affect protein digestion in the rumen in a number of ways including modification of digestibility and alteration of the microbial ecosystem (See Rogers *et al.*, 1982; Kandylis, 1984). Recent reviews (e.g., Clark *et al.*, 1992, Stern *et al.*, 1994) describe the influences of various dietary factors on the amount of microbial protein reaching the duodenum.

Protein flowing from the rumen is further modified through the inclusion of mucosal secretions and desquamated epithelial tissue associated with the digestive process. However, the precise magnitude of this endogenous fraction is difficult to determine owing to the difficulty of separating it from other proteins.

Ingested protein is, hence, subject to considerable modification in the rumen and, in consequence, the quantity and quality of protein entering the abomasum and available for digestion and absorption bears little resemblance to the quantity and quality of protein ingested by the ruminant.

1.2.3. Post-ruminal digestion of protein

Microbial cells and undegraded dietary protein together with small amounts of peptides and free amino acids pass with the digesta from the rumen-reticulum through the omasum and abomasum to the small intestine. Digestion of protein in the abomasum and small intestine appears to be the same for ruminants as non-ruminants except for the slow neutralisation of digesta in the small intestine and the abundance of pancreatic ribonuclease (Bergen, 1978).

The proteases found in the lower gut of the ruminant are listed in table 1-1. The proteases are secreted as inactive proenzymes or zymogens that become effective enzymes

Table 1-1. Proteases of post-ruminal gut in ruminants.

Protease	Origin or source	Site of activity	Protease type
Pepsin	Abomasum	Abomasum	Endo
Trypsin	Pancreas	Small intestine	Endo
Chymotrypsin	Pancreas	Small intestine	Endo
Elastase	Pancreas	Small intestine	Endo
Carboxypeptidase A	Pancreas	Small intestine	Exo
Carboxypeptidase B	Pancreas	Small intestine	Exo
N-terminal exopeptidases	Small intestinal mucosa	Small intestine	Exo

after activation. There are two general classes of abomasal and pancreatic proteases. The endopeptidases such as pepsin, trypsin, chymotrypsin, and elastase attack the interior peptide bonds between adjacent amino acids and differ functionally from each other only in their specificity for particular amino acids. The principal exopeptidases are the carboxypeptidases, which act on terminal peptide bonds at the carboxyl end of protein chains (Gray and Cooper, 1971).

Proteins entering the lower gut first become subjects of hydrolysis by the abomasal protease, pepsin, which is activated from pepsinogen by acid, and has broad specificity for peptide bonds involving aromatic L-amino acids (Gray and Cooper, 1971; Castro, 1991).

Digesta reaching the small intestine stimulate the release of secretin and cholecystokinin (CCK), which in turn causes the pancreas to secrete bicarbonate and enzymes into the intestinal lumen (Castro, 1991). Trypsin is secreted from the pancreas into the duodenum as the inactive zymogen, trypsinogen, and is activated at pH 7 by autocatalysis by trypsin, enterokinase and thrombin (Keil, 1971). It has a very narrow specificity compared with pepsin, hydrolysing only peptide bonds containing the carboxyl group of arginine or lysine (Keil, 1971).

Chymotrypsinogen, being very similar in structure to trypsinogen (Keller, 1968), is activated by relatively small amounts of trypsin (one part in 70) (Nordstrom and Dahlqvist, 1970). Chymotrypsin hydrolyses peptide bonds adjacent to carboxyl groups of aromatic and large hydrophobic amino acids such as tyrosine, tryptophan, phenylalanine, leucine and methionine (Bergen, 1978; Castro, 1991).

Proelastase, the inactive zymogen, becomes activated by trypsin to elastase, an enzyme with a remarkably similar structure to trypsin and chymotrypsin. Elastase is the only protease that can attack elastin and has broad specificity against interior peptide bonds involving neutral aliphatic amino acids yielding products with neutral amino acids at C-terminal end (Hartsuck and Lipscomb, 1971).

Exopeptidases can be divided into two categories, carboxypeptidases and aminopeptidases. Activation of bovine carboxypeptidase A involves extensive degradation of its parent zymogen, procarboxypeptidase A, by trypsin. Once secreted from the pancreas into the duodenum, carboxypeptidase A acts on peptides with aromatic and neutral aliphatic amino acids at the C-terminal end of substrates. Carboxypeptidase B is also activated by trypsin but with much simpler steps than carboxypeptidase A and it only attacks peptides with C-terminal lysine or arginine residues.

The intestinal mucosa secretes a number of peptidases commonly referred to as aminopeptidases or N-terminal exopeptidases, which release free amino acids and small peptides from the N terminus of the peptide substrate. Detailed information on the peptidases will be dealt with in the next section.

The pancreatic proteases require a pH of at least 5 for activation and their pH optimum for proteolytic activity is between 7 and 8. In ruminants, the duodenum and upper jejunum are still notably acid (pH 2 to 3) and the pH does not reach 6 to 7 until the middle to lower jejunum (Lennox *et al.*, 1968; Ben-Ghedalia *et al.*, 1974). This slow neutralisation of digesta passing from the abomasum to the small intestine can be related to the low bicarbonate content of pancreatic juice in the ruminant (Taylor, 1962). Indeed, in sheep, considerable proteolysis in the duodenum is still due to the gastric protease, pepsin. Optimal carboxypeptidase activity does not occur until the middle jejunum in sheep (Ben-Ghedalia *et al.*, 1974). The peak activities of N-exopeptidases and dipeptidases were found in the mid ileum (Ben-Ghedalia *et al.*, 1974)

Ruminal microorganisms contain 10 to 20 % of their N content as nucleic acids (Ellis and Pfander, 1965). The breakdown of the nucleic acids to mononucleotides is achieved by deoxyribonuclease (DNAse) and ribonucleases (RNAses).

1.2.4. Absorption of the end products of protein digestion

Polypeptides and protein fragments, resulting from the actions of gastric and pancreatic enzymes, become subjects of further hydrolysis by various intestinal mucosal peptidases prior to absorption across the intestinal epithelium into the circulatory system.

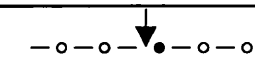
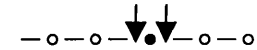
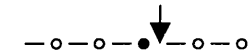
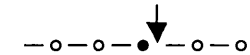
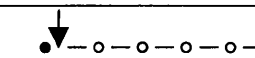
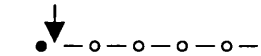
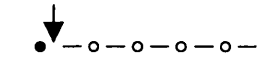
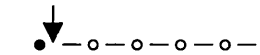
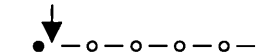
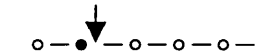
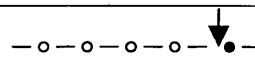
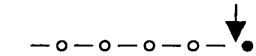
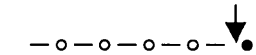
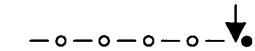
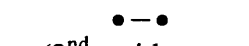
In spite of extensive studies and technical advancement in past decades in the area of intestinal peptidases, there are still new types of peptidases being discovered (Shneider *et al.*, 1997). Hence, the complete details of the mechanisms involved in this final stage of protein digestion by the actions of intestinal peptidases are still to be explored. Furthermore, there is relatively limited knowledge on intestinal digestion and absorption of protein in ruminant animals compared with simple-stomached animals, although it is generally assumed that the basic mechanisms are similar. Therefore, the information described here is mostly from simple-stomached animals.

The intestinal peptidases exist on the surface of the brush border of the intestinal epithelium as well as within their columnar cells, and their physiological functions are distinctively different (Gray and Cooper, 1971; Adibi and Kim, 1981; Gray, 1989).

There are several peptidases integral to the brush border membrane whose active sites, positioned external to the enterocyte's surface membrane, interact with oligopeptides while they are still within the lumen (table 1-2). These peptidases play a pivotal role in the interfacial digestion prior to final transport of the amino acids and small peptides (Gray, 1989).

Among the brush border peptidases, aminooligopeptidase, commonly called aminopeptidase N, has been most thoroughly characterised (Adibi and Kim, 1981; Gray, 1989). The aminooligopeptidase has a broad specificity for certain amino acid side chains at the N-terminus of the peptide substrate and sequentially removes single amino acid

Table 1-2. Peptidases of brush border membrane of intestinal cells (After Woodley, 1994).

Class	Name	Specificity
Endopeptidases	Endopeptidase-24.11 (EC 3.4.24.11)	 hydrophobic
	Endopeptidase-24.18 (EC 3.4.24.18)	 aromatic
	Endopeptidase-3 (EC ?)	 Arg-Arg ?
	Enteropeptidase (EC 3.4.21.9)	 $(Asp)_4$-lys
Exopeptidases : Amino-terminus	Aminopeptidase N (EC 3.4.11.12)	 many
	Aminopeptidase A (EC 3.4.11.7)	 Asp, Glu
	Aminopeptidase P (EC 3.4.11.9)	 Pro
	Aminopeptidase W (EC 3.4.11.16)	 Tryp, Tyr, Phe
	γ-glutamyl transpeptidase (EC 2.3.2.2)	 γ-glutamic acid
	Dipeptidyl peptidase IV (EC 3.4.14.5)	 Pro, Ala
Exopeptidases : Carboxy-terminus	Peptidyldipeptidase A (EC 3.4.15.1)	 Many, but especially His-leu
	Carboxypeptidase P (EC 3.4.17.16)	 Pro, Gly, Ala
	Carboxypeptidase M (EC 3.4.17.12)	 Lys, Arg
	γ-glutamyl carboxypeptidase (EC 3.4.19.9)	 $(\gamma\text{-glutamic acid})_n$
Dipeptidase	Microsomal dipeptidase (EC 3.4.13.11)	 Many (2^{nd} residue may be D configuration)

residues, yielding the free amino acids and smaller oligopeptides for transport (Adibi and Kim, 1981; Gray, 1989).

Besides the aminooligopeptidase, another aminopeptidase (dipeptidyl amino-peptidase) and two carboxypeptidases (carboxypeptidase P and dipeptidyl carboxy-peptidase) have been shown to be concentrated in the brush border membrane of the enterocyte and to have a sufficiently high affinity and hydrolytic capacity to perform an important physiological role in terminal peptide digestion (Bella *et al.*, 1982; Morita *et al.*, 1983; Yoshioka *et al*, 1987 and Yoshioka *et al.*, 1988). As shown in table 1-2, the peptidases cleave the first or second peptide bond at either the N-terminus or the C-terminus of the oligopeptide substrate, thereby removing a free amino acid or dipeptide.

Dipeptidyl aminopeptidase, commonly known as dipeptidyl aminopeptidase IV, acts on peptides with a free α-amino group, cleaving bonds involving the carboxyl group of either Pro or Ala and releasing the corresponding dipeptides X-Pro and X-Ala (Adibi and Kim, 1981). Since all of the other aminopeptidases are blocked by the presence of a Pro residue when it is at the N-terminal or penultimate position of the peptide substrate, the dipeptidyl aminopeptidase plays a pivotal complementary role in the surface digestion of dietary peptides.

Similarly, dipeptidyl carboxypeptidase cleaves a proline-containing dipeptide from the C-terminus. Although it has fairly broad specificity for amino acids at the C-terminus, its hydrolytic rate is enhanced two- to five-fold when the peptide chain terminates in a Pro residue (Yoshioka *et al.*, 1988). The carboxypeptidase P removes a single amino acid residue from the C-terminus while its efficiency of hydrolysis increases when a proline residue is located at the penultimate position (Yoshioka *et al.*, 1988).

Overall, brush border peptidases appear to break down oligopeptides released from pancreatic protease action into both free amino acids and smaller di- and tripeptides in a concerted manner to promote efficient surface hydrolysis of oligopeptides before the

transport of amino acids and small peptides across the brush border by specific intra-membrane macromolecular carriers of the enterocyte.

Amino acid transport in the small intestine

The duodenal, jejunal, and ileal regions of the small intestine appear to have abilities to absorb amino acids, although, in the ruminant, the more distal region of the small intestine, the ileum, has the major capacity for amino acid absorption (Johns and Bergen, 1973; Phillips *et al.*, 1979; Wilson and Webb, 1990).

The uptake of amino acids across the small intestinal epithelium involves two stages: uptake across the brush border membrane, and exit into the blood across the basolateral membrane both of which can be achieved by either simple diffusion or mediated transport (Matthews, 1991a). Simple diffusion is not generally thought to be a major mechanism in the transepithelial transport of amino acids, although results *in vitro* sugest that it may be more important for lysine and methionine (Moe *et al.*, 1987; Wilson and Webb, 1990). Mediated transport involves a 'special mechanism (carrier)' which aids transport across the membrane and is specific for a group of structurally related substrates, hence is subject to competitive inhibition by other substrates sharing the same mechanism (Matthews, 1991a). There are two types of mediated transport, one is facilitated diffusion (Na^+-independent) and the other is active transport (Na^+-dependent). Facilitated diffusion is dependent for its operation on the presence of a concentration difference of solute across the membrane and, naturally, takes place down the concentration gradient whereas active transport is capable of transporting a substrate across a membrane against an electrochemical gradient and is, directly or indirectly, driven by metabolic energy consumption which is actually associated with the continuous outward flux of Na^+ from the mucosal cells owing to the activity of a Na^+ / K^+ 'pump'(Webb, 1990; Matthews, 1991a).

Although the precise number of transporters for amino acids present in the small intestine is not known, there are several transport systems that have been defined and characterised in both the brush border membrane and the basolateral membrane, based on substrate preference and by kinetic and inhibition analysis measurements (Christensen, 1984). The major transport systems found in the small intestine and their substrate specificities are listed in table 1-3. It is noteworthy that species differences of amino acid transport system in the small intestine exist even among simple-stomached animals (Munck and Munck, 1994) and that recent advances in the identification and sequencing of specific transport proteins by methods of molecular biology have opened new prospective opportunities for more detailed understanding of amino acid transport systems in the small intestine (McGivan and Pastor-Anglada, 1994).

Peptide transport in the small intestine

Since Newey and Smith (1959, 1960) provided the first convincing evidence indicating the possibility of peptide absorption, much effort has been committed to advance our knowledge of peptide absorption in the small intestine, and it is now generally accepted that small peptides, mostly di- or tripeptides, are absorbed across the intestinal epithelium in simple-stomached animals (see Matthews, 1975; Adibi and Kim, 1981; Gardner, 1982; Gardner, 1984; Silk *et al.*, 1985; Matthews, 1991b) and ruminant animals (see Webb, 1986; Webb, 1990; Webb and Bergman, 1991; Webb *et al.*, 1992; Webb and Matthews, 1998).

There is evidence both in humans and in experimental animals, that dipeptides disappear at significantly slower rates in the ileum than in the jejunum (Adibi, 1971; Crampton *et al.*, 1973; Schedl *et al.*, 1979) but it is not clear whether it is the same in the ruminant. A number of recent studies (Webb *et al.*, 1992; Matthews and Webb, 1995; Matthews *et al.*, 1996) suggest that the forestomachs may have an ability to absorb

Table 1-3. Amino acid transport systems in the small intestine of higher animals (Modified from Barker and Ellory, 1990; McGivan and Pastor-Anglada,1994).

System		Specificity
Na^+-dependent systems	A	Small aliphatic amino acids; methyl-AIB
	ASC	Small aliphatic amino acids; not methyl-AIB
	B	Broad specificity; most neutral amino acids
	$B^{0,+}$	Broad specificity; most neutral and basic amino acids
	X_{AG}^-	Glutamate, aspartate
	β	β-Alanine, taurine
Na^+-independent systems	L	Mainly branched-chain and aromatic amino acids
	y^+	Lysine, histidine, arginine
	$b^{0,+}$	Neutral and basic amino acids

significant amounts of peptide. However, this needs to be confirmed by further investigations *in vivo*.

The physiological significance of peptide transport in amino acid metabolism was highlighted by a number of early studies in which peptides were absorbed from the intestinal lumen more rapidly than equivalent mixtures of free amino acids (Adibi and Phillips, 1968; Craft *et al.*, 1968; Adibi, 1971; Burston *et al.*, 1972). Furthermore, a number of studies *in vivo* and *in vitro* suggested that absorption of intact peptides may be quantitatively significant in the supply of amino acids to tissues for protein synthesis (Gardner, 1975; Koeln and Webb, 1982; McCormick and Webb, 1982; Danilson *et al.*, 1987; Koeln *et al.*, 1993). However, the quantitative nutritional significance of the absorbed peptides is still the subject of intense debate owing to the lack of reliable and reproducible methods for peptide measurement in the plasma (Seal and Parker, 1998; Backwell, 1998).

Although the existence of a transport process for intact peptides in the brush border membrane of intestinal epithelial cells has been known for almost three decades, it is only recently that the molecular nature of the protein responsible for the transport process in the small intestine has been elucidated (Miyamoto *et al.*, 1991; Saito *et al.*, 1993; Tamai *et al.*, 1994; Tamai *et al.*, 1995).

There seems to be a single H^+-gradient-dependent peptide transporter, PEPT 1, in the small intestine which has high capacity for di- and tripeptides of widely differing structure (Gray, 1989; Leibach and Ganapathy, 1996). It requires at least two, and no more than three, amino acid residues covalently linked by a peptide bond with an unsubstituted α-NH_2 group at the N-terminal position (Matthews, 1975), and requires an energising or driving force (H^+) generated and maintained by the combined action of the Na^+-H^+ exchanger in the brush border membrane and the Na^+-K^+-adenosine triphosphatase (ATPase) in the basolateral membrane (Leibach and Ganapathy, 1996). There is no second

peptide transporter identified in the small intestine at present although the presence of multiple transport systems or several subtypes of a single transporter can not be ruled out (Muranushi *et al.*, 1994; Yuasa *et al.*, 1994).

The peptide transport system exists both in the brush border membrane and the basolateral membrane, although some evidence indicates that the transport systems present in these two membranes may be distinct (Saito and Inui, 1993; Thwaites *et al.*, 1993a; Thwaites *et al.*, 1993b). Peptides absorbed by the transporter in the brush-border membrane of intestinal enterocytes are generally assumed to be extensively hydrolysed by numerous cytoplasmic peptidases and only less than 10 % of them can reach the blood intact (Shoaf *et al.*, 1976; Adibi and Kim, 1981; Matthews, 1991b). However, non-mediated absorption of nutritionally significant amounts of oligopeptides has been observed through a paracellular pathway ('leaky' tight junctions) in enterocytes (Atisook and Madara, 1991) which could have at least pharmacological if not nutritional importance.

1.3. PROTEIN METABOLISM IN THE DAIRY COW

The fate of absorbed amino acids (and peptides) can be either catabolic losses, including digestive secretions and tissue oxidation, or anabolic protein gain such as muscle and milk. Among the splanchnic tissues, the gastrointestinal tract has the highest rate of amino acid turnover owing to a heavy demand from mucosal cell turnover and the continual secretion of digestive enzymes and mucus glycoproteins. This high turnover rate, together with the use of amino acids as energy substrates within the mucosa, probably accounts for the poor recovery of individual amino acids across the portal drained viscera (PDV). Tagari and Bergman (1978) reported that between 30 and 80 % of amino acids disappearing from the lumen do not appear in the portal vein in sheep. Although a more

recent study (MacRae *et al.*, 1995) reported that recovery of absorbed essential amino acids across the mesenteric drained viscera was apparently complete, there were still 33 to 45 % of essential amino acids disappearing from the small intestine that did not reach the portal vein in sheep. Furthermore, Guerino *et al.* (1991) reported that only 28 % of post-ruminal casein nitrogen appeared in the portal blood as α-amino nitrogen in steers. Therefore, the pattern of amino acids available to the liver and peripheral tissues differs significantly from that of absorbed amino acids (Lobley *et al.*, 1980).

Absorbed amino acids and peptides entering the portal circulation reach the liver where a large part of the amino acids are removed through catabolic processes and, as a by-product, ammonia is released most of which is converted into urea. In lactating dairy cows, 42 % of net PDV appearance of α-amino N and 38 % of net PDV appearance of total amino acid were subsequently removed on a net basis by the liver (Reynolds *et al.*, 1988; Cohick, 1989). The rest of the amino acids undergo a great variety of reactions such as the synthesis of protein, formation of peptide hormones, and production of special compounds like detoxification derivatives, ketone bodies, nonpeptide hormones, glutathione and many others. In addition to supplying building blocks for protein, amino acids, especially alanine, glycine, serine and glutamine, also supply a large proportion (up to 30 %) of the glucose needed by ruminant animals (Wolff and Bergman; 1972, Huntington and Eisemann; 1988).

1.3.1. Uptake and utilisation of amino acids and peptides by the mammary gland

The lactating mammary gland extracts large amounts of free amino acids from the circulation in order to meet the requirements of milk protein synthesis. This high demand for free amino acids by the mammary gland is demonstrated by studies showing substantial

arteriovenous amino acid concentration differences across the mammary gland (Fleet and Mepham, 1985).

Although relatively few studies have been carried out to identify amino acid transport systems in the mammary gland compared with other organs (i.e. small intestine, liver, kidney and placenta), it appears that amino acids are taken up by mammary secretory cells via a large array of distinct transport systems that are similar to the amino acid transport systems found in other epithelial cells (see previous section, table 1-3). However the amino acid transport systems in the mammary gland are distributed differently to meet a large requirement for the anionic amino acid, L-glutamate (system X_{AG}) and transport systems with low-specificity and high-capacity (system L and A) seem to play a major role in the mammary gland (see Shennan, 1998).

To study quantitative relationships between substrate supply and milk synthesis, *in vivo* measurements of arteriovenous differences across a gland and the transfer of radioactivity from plasma precursors to milk constituents, or a combination of the two techniques have been used most widely. Amino acid requirements of the mammary gland for metabolism and milk synthesis can be estimated quantitatively by using the same techniques and, based on this estimation, amino acids that are in shortest supply relative to demand (Met, Lys, Phe and His), have been proposed to limit milk protein synthesis (Derrig *et al.*, 1974; Clark *et al.*, 1977; Oldham, 1981).

The uptake of most essential amino acids, especially branched-chain amino acids, by the mammary gland is in excess of the output in milk. However, some studies of net amino acid transfer across the mammary gland have indicated that, for certain amino acids (Met, His, Phe and Trp), the amount disappearing from the circulation is insufficient to account for their output in milk protein (Bickerstaffe *et al.*, 1974; Metcalf *et al.*, 1994) suggesting that other sources of amino acids such as peptides and proteins must be involved in their supply. Indeed, indirect evidence for the utilisation of peptides for milk

protein synthesis in the lactating dairy goat *in vivo* have been obtained recently using arteriovenous difference and dual-labelled tracer techniques together with an isotope kinetic technique (Backwell *et al.*, 1994; Backwell *et al.*, 1996), although methodological problems still make it difficult to obtain direct evidence of peptide uptake across the tissue bed.

Despite the strong possibility of the utilisation of peptides by the mammary gland, there is not much information available on how peptide uptake by the mammary gland occurs. However, Shennan *et al.* (1998) recently demonstrated that the uptake of two hydrolysis-resistant dipeptides, D-[^{3}H]phe-L-glu and D-[^{3}H]phe-L-gln, by the perfused lactating rat mammary gland is very low even under conditions designed to maximise uptake of the radiolabelled compounds, and that anionic dipeptides *trans*-stimulate D-aspartate efflux from mammary tissue via the high-affinity anionic amino acid carrier suggesting the dipeptides were hydrolysed extracellularly followed by uptake of the constituent free anionic amino acids via the mammary tissue high affinity, Na-dependent anionic amino acid carrier.

1.3.2. Milk production responses to post-ruminal supplements of casein

Attempts have been made successfully to improve milk production of dairy cows by supplementing the diet with protein (Van Horn *et al.*, 1979; Cressman *et al.*, 1980; Cowan *et al.*, 1981; Holter *et al.*, 1982; Macleod *et al.*, 1984; Chamberlain *et al.*, 1992a). However, the mechanisms underlying the responses are not clear for a number of reasons, including accompanying increases in feed intake and fibre digestibility leading to an increased supply of metabolisable energy (ME), as well as the uncertainty over the degradability of dietary protein in the rumen.

To overcome the difficulty of interpreting milk production responses to protein supplements, the infusion of proteins and amino acids direct into the abomasum or the duodenum has been used. In most of these experiments, casein was chosen as a convenient, soluble and well-balanced source of amino acids.

In most studies, post-ruminal administration of casein increased milk production of lactating dairy cows (see Rulquin, 1982; Choung and Chamberlain, 1993a and Huhtanen *et al.*, 1997) and of lactating goats (Ranawana and Kellaway, 1977a; Ranawana and Kellaway, 1977b) with very few exceptions (Hale and Jacobson, 1972 and Vik-Mo *et al.*, 1974). It appears that the milk production response to post-ruminal administration of casein is greater in the high producing cow, and tends to be greater at lower concentrations of protein in the diet.

These increases in milk production in response to casein infusion might not be unexpected since it is the major milk protein and therefore should offer an ideal pattern of amino acid for synthesis of milk protein. However, in some studies, there were substantially greater incremental increases in the output of energy in milk than was provided by the casein infusions suggesting mobilisation of body tissue or a repartitioning of ME use in favour of the mammary gland (Ørskov *et al.*, 1977; Konig *et al.*, 1984; Whitelaw *et al.*, 1986; Choung and Chamberlain, 1992b). This altered partitioning of nutrient use between the mammary gland and adipose tissue might have resulted from changes in major metabolic hormones like GH and insulin (Choung and Chamberlain, 1992c; Oldham, 1994).

Furthermore milk production responses to abomasal infusion of soya protein isolates (SPI) were markedly smaller than to casein and the addition of amino acids to the SPI in attempt to make it equivalent to casein in the supply of total amino acids and all the individual essential amino acids has failed to make up the differences between two protein sources (Choung and Chamberlain, 1992a; Choung and Chamberlain, 1992c). These

findings suggest that the superiority of casein over SPI cannot be due solely to its total or essential amino acid content but also to additional factors associated with the characteristics of digestion and the patterns of absorption of amino acids and peptides from the protein sources.

In a further series of experiments, Choung and Chamberlain (1993b, 1995a, b) have also reported that the nature of the milk production response to abomasal infusion of various hydrolysates of casein was related to the proportion of peptide-bound amino acids in the infusates. There were clear differences between abomasal infusions of caseinate as opposed to hydrolysed casein or corresponding mixture of free amino acids (FAA) in their effects on the yield of milk fat and, at some levels of infusion, caseinate is superior to hydrolysates or FAA in its effects on the output of milk protein. These results together with previous findings clearly indicate the possibility of important effects of peptides produced during digestion of casein on the metabolism of the lactating dairy cow.

Another interesting finding in these experiments is that the pattern of milk production response depended on the level of infusion. For example, differences in effects on the output of milk protein were evident only at the lower level (146 g / d) of input where free amino acid mixtures supported a smaller increase in protein yield than did the corresponding level of caseinate. Similarly, the increases in the concentration of fat in the milk in response to abomasal infusion of the hydrolysate in comparison with sodium caseinate disappeared completely at the highest level (440 g / d) of infusion (Choung and Chamberlain, 1995a). Such effects would probably not be detected using the much larger amounts of casein that are typical of most experiments reported in the literature.

It is not easy to explain why the form in which amino acids enter the post-ruminal gut should influence the response of milk production. However, Choung and Chamberlain (1995a) suggested that the faster absorption of amino acids in peptide-bound form compared with free amino acids might underlie the mechanism, presumably via endocrine

effects; alternatively, biologically active peptides produced from casein during the enzymatic hydrolysis might be involved.

1.4. BIOACTIVE PEPTIDES FROM MILK PROTEIN

Milk contains a large number of bioactive peptides with various biological activities. These include natural bioactive peptides such as epidermal growth factor (EGF), transforming growth factor (TGF), nerve growth factor (NGF), insulin and insulin-like growth factor I and II (IGF-I and IGF-II) which may have significant influences on suckling neonates (table 1-4). Many peptides have also been identified encoded into the primary structures of milk proteins, which can be released by enzymatic hydrolyses when ingested (table 1-5). Some of these bioactive peptides have marked influences on gastrointestinal functions. For example, phosphopeptides enhance gastrointestinal absorption of calcium, and β-casomorphins inhibit gastrointestinal contraction and fluid secretion (Schlimme and Meisel, 1995). During the past two decades, these bioactive peptides have received much attention as potential physiological modulators during the gastrointestinal digestion of milk and, among the peptides, casomorphins have been investigated and characterised most extensively.

1.4.1. β–Casomorphins : Discovery and chemical and biological identity

Brantl *et al.* (1979) first described successful isolation of a material from a peptone digest of bovine β-casein that displayed opioid activity in the guinea pig ileum longitudinal muscle-myenteric plexus preparation. They named the material as β-casomorphin which implies 'morphin-like-material from β-casein'. In the accompanying paper (Henschen *et al.*, 1979), it was shown that the material is a pure heptapeptide with the sequence 'Tyr-

Table 1-4. Bioactive proteins and peptides as natural ingredients of milk (After Schlimme and Meisel, 1995).

Thyrotropin-releasing hormone (TRH)
Luteinising hormone-releasing hormone (LHRH)
Somatostatin
Gastrin-releasing peptide (GRP)
Calcitonin
Adrenocorticotropic hormone (ACTH)
Insulin
Growth factors (e.g. EGF)
Relaxin
Prolactin
Thyroid stimulating hormone (TSH)
Lysozyme
Lactoperoxidase
Lactoferrin
Transferrin
Immunoglobulins (IgA, IgM, IgG)
Enzymes (e.g. plasmin)

Table 1-5. Bioactive peptides derived from milk proteins (After Schlimme and Meisel, 1995).

Bioactive peptides	Protein precursor	Bioactivity
α-Casomorphins	α-Casein	Opioid agonist
β-Casomorphins	β-Casein	Opioid agonist
α-Lactorphin	α-Lactalbumin	Opioid agonist
β-Lactorphin	β-Lactoblobulin	Opioid agonist
Lactoferroxins	Lactoferrin	Opioid antagonists
Casoxins	χ-Casein	Opioid antagonists
Casokinins	α-, β-Casein	Antihypertensive
Casoplatelins	χ-Casein, Transferrin	Antithrombotic
Immunopeptides	α-, β-Casein	Immunostimulants
Phosphopeptides	α-, β-Casein	Mineral carriers

Pro-Phe-Pro-Gly-Pro-Ile' which corresponds to the 60th to 66th residues of the β-casein A2 sequence (figure 1-3).

C-terminally shortened peptides (β-casomorphin-4, β-casomorphin-4-amide and β-casomorphin-5) from the heptapeptide (β-casomorphin-7) and its pro-form (β-casomorphin-11) have been found to possess opioid activity with β-casomorphin-5 and β-casomorphin-4-amide being most potent agonists (Henschen *et al.*, 1979; Brantl *et al.*, 1981; Chang *et al.*, 1981; Brantl *et al.*, 1982; Meisel and Frister, 1988, 1989). The identical sequences of bovine β-casomorphin-7 also appear in the peptide chains of buffalo (Petrilli *et al.*, 1983 and Petrilli *et al.*, 1987) and ovine (Richardson and Mercier, 1979) β-caseins but not in that of human (Greenberg *et al.*, 1984) and rat (Blackburn *et al.*, 1982). However, similar peptides (i.e. human β-casomorphin-8 [Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro]) have been isolated from human β-casein (Brantl, 1984; Greenberg *et al.*, 1984) and their opioid activities have been demonstrated (Brantl, 1984).

Since their discovery, β-casomorphins and their immunoreactive material have been identified in an enzymatic digest of bovine casein *in vitro* (Chang *et al.*, 1985; Svedberg *et al.*, 1985), fermented milk (Matar and Goulet, 1996), milk from a woman with postpartum psychosis (Renlund *et al.*, 1993), plasma of women during pregnancy and after delivery (Koch *et al.*, 1988), the small intestine contents of adult humans (Svedberg *et al.*, 1985) and minipigs (Meisel, 1986), and the plasma of newborn calves (Umbach *et al.*, 1985) after ingestion of a casein diet or milk.

The presence of tyrosine residues at the N-terminal position and of another aromatic residue, Phe or Tyr, in the third or fourth position is a common feature among opioid peptides (i.e. enkephalin, endorphins, and dynorphin). Indeed, alterations at the N-terminal of β-casomorphin-7 (e.g. acetylation) completely abolished the opioid activity of the

41 50

Thr – Glu – Asp – Glu – Leu – Gln – Asp – Lys – Ile – His – Pro – Phe – Ala – Gln – Ser – Leu –

60 70

Val – **Tyr – Pro – Phe – Pro – Gly – Pro – Ile** – Pro – Asn – Ser – Leu – Pro – Gln – Asn – Ile –

His (Variants C, A1 and B)

80 90

Pro – Pro – Leu – Thr – Gln – Thr – Pro – Val – Val – Val – Pro – Pro – Phe – Leu – Gln – Pro –

100

Glu – Val – Met – Gly – Val – Ser – Lys – Val – Lys - Glu

Figure 1-3. β-casomorphin sequences (bold typed region) in the primary structure of bovine β-casein A2 variant.

peptide indicating the importance of the N-terminal tyrosine residue for the biological activity of the peptide.

β-Casomorphins have an intensely bitter taste that is a characteristic of hydrophobicity of a peptide. This high hydrophobicity of β-casomorphins together with the fact that the peptides exist in trace amounts in complex peptide mixtures contributes to significant analytical difficulties in identifying the peptides in biological fluids. Recent advances in analytical techniques have led to the development of methods to identify β-casomorphins and their analogues in mixed samples by HPLC (Meisel, 1993), RIA (Teschemacher *et al.*, 1980 and Chang *et al.*, 1985) and Enzyme-Linked Immunosorbent Assay (ELISA) (Yannakis and Ozimek, 1998) in conjunction with appropriate concentration steps (i.e. solid phase extraction) and production of antisera for the peptides (see Schlimme and Meisel, 1995 for more details). Highly purified β-casomorphins can also be obtained by solid phase peptide synthesis, which is easily carried out by the polyamide continuous flow method on an automatic peptide synthesiser combined with FMOC chemistry (Meisel and Schlimme, 1994)

Many attempts have been made to make β-casomorphins more stable from attack of various enzymes as well as more potent and specific to an opioid receptor type. These usually involve modification by replacing the natural L-amino acids by their D-analogues or by D-pipecolic acid (Schnittler *et al.*, 1990) and amidation of their C-terminal amino acids (Chang *et al.*, 1981).

1.4.2. Pharmacophysiological actions of β-Casomorphins

Since β-casomorphins appear to be highly resistant to proteolytic enzymes, it had been speculated that they might reach the blood circulation and the opioid receptors in brain intact, modulating physiological function via the opioid system (Brantl and

Teschemacher, 1979). However, it soon became evident that the dipeptidyl-peptidase IV (DP-IV), the enzyme specific for cleaving dipeptide fragments (X-Pro) from the N-terminus of peptides, was able to cleave the β-casomorphins (Kreil *et al.*, 1983). Consequently, β-casomorphins are rapidly degraded in bovine or rat plasma with half-lives of less than 10 minutes (Kreil *et al.*, 1983). Since the same peptidase activity is present in the brush border of the small intestine (see previous section), it seems unlikely that natural β-casomorphins are absorbed intact by the alimentary tract.

However, contrary to the common belief of systemic unavailability owing to digestion, orally-ingested simple proteins are known to exert biological effects both on the central and peripheral nervous systems (Staub *et al.*, 1978; Masson et al., 1979; Morley, 1982; Kastin *et al.*, 1984). Furthermore, incubation of buffalo β-casein with proteolytic enzymes (Petrilli *et al.*, 1984; Caporale *et al.*, 1985) and *in vivo* digestion of the milk protein casein in minipigs (Meisel, 1986) leads to the formation of β-casomorphin precursors (i.e. β-casomorphin-11). Therefore, in spite of their sensitivity to DP-IV, it could be suggested that these peptides can survive in blood and reach their putative brain receptors in the peptidase-resistant precursor form (i.e. procasomorphins). Alternatively, β-casomorphins could be available systemically through a paracellular pathway ('leaky' tight junctions) in enterocytes (see previous section).

Based on the binding affinity of β-casomorphins to opioid receptor subtypes, it appears they are more selective ligands of the μ-type opioid agonists (Brantl *et al.*, 1981; Grecksch *et al.*, 1981; Koch *et al.*, 1988). Opioid activity of β-casomorphins has also been assessed in isolated tissue preparations like guinea-pig ileum and mouse vas deferens. The guinea-pig ileum has both opioid-containing neurons and receptors (Puig *et al.*, 1977; Collier *et al.*, 1981). In the electrically stimulated myenteric plexus-longitudinal muscle from the guinea-pig ileum, opiates bind with the μ-receptors. This binding leads to a

reduction in the ganglionic release of acetylcholine which in turn produces inhibition of the muscle contractions. The potency of opiates in this preparation correlates closely with their analgesic potency (Kosterlitz and Waterfield, 1975). The isolated mouse vas deferens preparation is considered to be more sensitive for δ-type opiate agonists (Lord *et al.*, 1977). The bovine β-casomorphins display greater opioid activity in the guinea-pig ileum as compared with mouse vas deferens (Lottspeich *et al.*, 1980) and this μ-type opioid activity of the β-casomorphins is well represented by many studies where they have been shown to elicit analgesia in rats (Brantl *et al.*, 1981; Grecksch *et al.*, 1981; Blass and Blom, 1996).

While their mechanism of action is still not clear, considerable research effort has been directed to determining the physiological role of β-casomorphins both in human and experimental animals. Opioid peptides are psychoactive compounds with hormonal and neurotransmitter activities and opioidergic systems are located in the central nervous, endocrine and immune systems (Schlimme and Meisel, 1995; Xu, 1998). This implies that, when an opioid peptide is systemically available, it can exert a range of various physiological and pharmacological effects.

The role of opioid peptides in gastrointestinal physiology (i.e. antidiarrhoeal effects) is well known (Daniel *et al.*, 1984; Miller *et al.*, 1984; Miller and Hirning, 1989). There is indirect evidence that β-casomorphins may regulate gastrointestinal motility. Intragastric administration of casein or casein hydrolysates reduces the amplitude and frequency of gastrointestinal contraction in dogs (Defilippi *et al.*, 1995) and in cattle (Kil and Froetschel, 1994), and slows gastric emptying and gastrointestinal transition in rats (Daniel *et al.*, 1990; Brust *et al.*, 1991). Such effects can be suppressed by pretreatment of the animals with naloxone or naltrexone, μ-type opiate receptor antagonists. It has also been reported that gastric acid secretion is inhibited in dogs with gastric fistulas by the administration of β-casomorphin-5, which is also antagonised by naloxone (Smagin *et al.*,

1983). However, no inhibiting activity of the β-casomorphin-5 was observed on gastrin secretion in the study.

A particularly interesting observation, in view of the potential nutritional importance of β-casomorphins, is that the L-[^{3}H] leucine uptake by rat jejunum *in vitro* was increased by 40 % when β-casomorphin-5 or an analogue of β-casomorphins (Tyr-Pro-Phe-Pyrrolidide) was coincubated at a physiological concentration (10^{-8} mol / L) indicating that the β-casomorphins may be external signals which regulate transport proteins and nutrient transfer via interaction with specific receptors (Ermisch *et al.*, 1989; Brust *et al.*, 1991).

Effects of β-casomorphins and their analogues on the secretion of pituitary, hypothalamic, pancreatic and gastric hormones have been reported. Plasma prolactin levels were increased in rats following systemic administration of bovine β-casomorphin-7 (Nedvidkova *et al.*, 1985). Intravenous administration of β-casomorphins increase the hypothalamic content of immunoreactive thyrotropin releasing hormone (Mitsuma *et al.*, 1984). Both oral and intravenous administration of β-casomorphins increases insulin secretion in dogs (Schusdziarra *et al.*, 1983a; Schusdziarra *et al.*, 1983c). In conscious dogs, β-casomorphin-4-amide, -5, -7 and casein peptone augmented the release of postprandial insulin after a liver extract-sucrose test meal. The augmented secretion was antagonised by oral naloxone (10 mg), suggesting the involvement of specific opioid-receptor mediated mechanism(s) (Schusdziarra *et al.*, 1983a). It was also reported by the same research group that intravenous administration of small doses of morphiceptin (5 nmol / kg) elicits significant inhibition of somatostatin-like immunoreactivity (Schusdziarra *et al.*, 1983b; Schick and Schusdziarra, 1985). Other hormones (i.e. CCK and pancreatic polypeptide) have also been found to be altered by the β-casomorphins (Schusdziarra *et al.*, 1983c; Schusdziarra *et al.*, 1984).

There has been considerable interest in the role of β-casomorphins in other aspects of pharmacophysiology in humans including food intake (Lin *et al.*, 1998), behaviour (Herrera-Marschitz *et al*, 1985; Maklakova *et al.*, 1993), physical dependence (Chang *et al.*, 1983), respiratory frequency (Hender and Hender, 1987) and cardiovascular function (Wei *et al.*, 1980) as well as neonatal immunostimulating activity (Parker *et al.*, 1984), neuronal differentiation (Sakaguchi *et al.*, 1998) and post-feeding calmness and sleep in infants (Sturner and Chang, 1988).

1.5. HORMONAL REGULATION OF NUTRIENT USE

Absorbed nutrients are partitioned differently among metabolic organs and tissues to accommodate successful execution of the dominant productive function according to physiological states (i.e. growth, pregnancy and lactation). Lactating animals, in particular, face tremendous tasks, especially in the early stage, to maximise available substrate to the mammary tissues in order to meet the requirement for milk synthesis. The partitioning of nutrients can be manipulated through altering the homeostatic and homeorhetic control of metabolism (both anabolic and catabolic) by hormones such as GH, insulin and glucagon (Bauman and Currie, 1980). This regulation of metabolism by the hormones occurs mainly by altering the kinetics of biochemical reactions, and by influencing substrate availability.

1.5.1. Nutrient partitioning by metabolic hormones in lactation

1.5.1.1. Insulin

There can be no doubt that insulin, secreted by the β-cells of the islets of Langerhans in the pancreas, is at the centre of metabolic regulation in ruminants as in other mammalian species (Bassett, 1975). However, it is important to remember that ruminant

animals on a normal diet absorb little dietary carbohydrate as glucose owing to the extensive microbial fermentation in the rumen (see previous section). Consequently glucose needs must be met by gluconeogenesis from other sources (i.e. propionate, lactate and amino acids) and acetate is the major precursor for *de novo* fatty acid synthesis (Brockman and Laarveld, 1986).

Insulinotrophic agents in ruminants also differ from those of nonruminants. It has long been known that VFAs, particularly propionate, are potent stimulants for secretion of insulin in the ruminant as well as glucose and amino acids. Increasing ruminal propionate infusion caused a linear increase in the net portal release of insulin in lambs (Gross *et al.*, 1990). However, intramesenteric infusions of propionate and glucose increased arterial and portal concentrations of insulin in non-lactating cows but not in lactating cows indicating the existence of interactions between pancreatic endocrine secretion and physiological state (Lomax *et al.*, 1979). Amino acids may also play an important role in postprandial insulin secretion in the ruminant as suggested by studies on sheep (Hertelendly *et al.*, 1970; Kuhara *et al.*, 1991). Interestingly, a recent paper by Lemosquet *et al.* (1997) reported a synergistic effect between glucose and amino acids on plasma insulin in lactating dairy cows when isocaloric amounts of amino acids or glucose alone or amino acids and glucose mixture were infused intravenously. Amino acid infusion induced lower and less prolonged plasma insulin release than glucose on an isocaloric basis.

Insulin has a multiplicity of effects in the nonruminant: it acts on a variety of tissues including muscle, adipose, liver and mammary gland, stimulating the uptake and utilisation of glucose by the peripheral tissues and inhibiting gluconeogenesis and glucose release from the liver as well as stimulating the uptake and incorporation of amino acids into protein. It has similar effects in ruminants. *In vitro* studies have reported that uptake of glucose by muscle (Jarrett *et al.*, 1974; Hay *et al.*, 1984) and adipose tissue (Vernon *et al.*, 1985) of sheep was facilitated by insulin although the responses were generally smaller

than those in rats and mice (see Brockman and Laarveld, 1986). Insulin, at low concentrations, reduced the hepatic removal of lactate, glutamine and glycerol, and also reduced the hepatic extractions of pyruvate and alanine at higher concentrations in sheep (Brockman, 1985).

The mammary gland, on the other hand, is regarded as insensitive to insulin in the ruminant. According to Laarveld *et al.* (1981), the removal of glucose by the bovine mammary gland is unchanged by insulin. Furthermore, insulin infusion at physiological level in conjunction with glucose, KCl-NaCl and amino acids failed to alter mammary uptake of glucose and essential amino acids in dairy goats (Tesseraud *et al.*, 1992). However, the AV difference across the mammary gland of lactating ewes for glucose was decreased progressively when insulin was increased suggesting that insulin may in fact affect mammary uptake of glucose (Leenanuruksa and McDowell, 1988).

The lack of responsiveness of tissues of ruminants to insulin may be linked to the pattern of feeding and digestion in the rumen which both minimise surges of nutrients, especially glucose, entering the portal vein thus decreasing the need for insulin to act rapidly to move nutrients into storage (e.g. glycogen and fat) although, in some circumstances where high concentrate diets are fed in high producing dairy cows, surges of propionate entering the portal vein can occur which result in increases in circulating insulin concentrations (Sutton *et al.*, 1988).

During early lactation serum insulin concentration falls in most species including cattle and sheep, then as lactation progresses and milk yields fall, serum insulin recovers (Cowie *et al.*, 1980). This fall in serum insulin has been attributed to the negative energy balance, which normally prevails at this stage. Figure 1-4 shows the typical energy input and output status of high producing dairy cows throughout lactation. Furthermore, the insulin responsiveness of mammary tissue is known to be low compared with other tissues in lactating ruminants (Brockman and Laarveld, 1986). Thus, higher insulin concentration

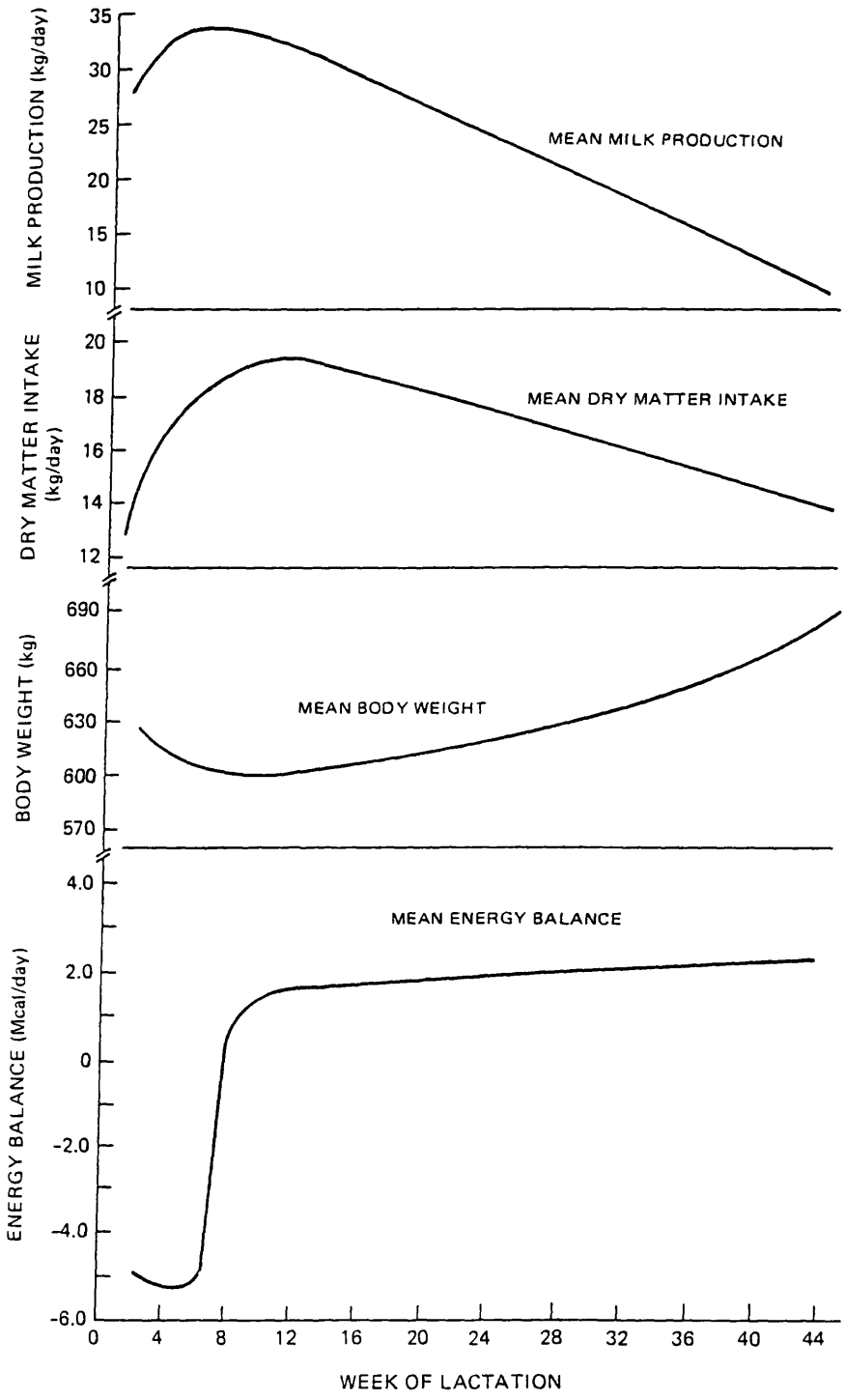


Figure 1-4. Trends in milk production, dry matter intake, body weight change and energy balance of dairy cows during lactation (After National Research Council, 1989).

could result in incorporation of available nutrients into non-mammary tissues, so reducing substrate availability for milk production. This selectivity of insulin action on different tissues and its lower concentration in lactating ruminants has led to speculation that insulin might have an adverse effect on milk production. Indeed, a comparison of circulating insulin in high- and low-yielding cattle revealed that during lactation the hormone circulated at significantly higher levels in the low-yielding cows (Hart *et al.*, 1978). This result is consistent with the finding that plasma insulin and milk yield are negatively correlated (Koprowski and Tucker, 1973).

It has long been hypothesised that insulin is responsible for 'low-fat milk syndrome' which occurs in cows consuming a high concentrate, low forage diet since insulin has a stimulatory effect on rates of lipogenesis and an inhibitory effect on rates of lipolysis in adipose tissue (Bauman and Elliot, 1983) but not in mammary tissue. This so called 'glucogenic-insulin theory' proposes that increased insulin release, resulting from feeding a high concentrate diet, preferentially channels nutrients to adipose tissue, leading to a shortage of nutrients at the mammary gland, thus causing milk fat depression (Jenny *et al.*, 1974; Brockman and Laarveld, 1986). In a study on the effects of feeding frequency in lactating cows, Sutton *et al.* (1988) found that more frequent feeding reduces the mean daily concentration of insulin and the depression in milk-fat concentration caused by feeding the low-roughage diets. They concluded that milk-fat depression on low-roughage diets with twice-daily feeding was due to a change in rumen VFA proportions accompanied by elevated plasma insulin concentrations.

However, insulin infusion studies have yielded no support for the role for insulin in milk fat depression (Schmidt, 1966; Thomas *et al.*, 1987). Furthermore, recent studies revealed that the mammary gland maintained a constant rate of milk fat synthesis despite the substantial challenges imposed by hyperinsulinemia (McGuire *et al*, 1995; Griinari *et al.*, 1997a). It was proposed that the changes in body fat accretion and adipose tissue

metabolism in lactating ruminants are consequences of a more positive energy balance caused by the reduced output of milk fat and the higher net energy intake typically associated with high concentrate diets (Griinari *et al.*, 1997a). However, underlying mechanisms of the milk fat depression are still not clear.

Recently, studies with the hyperinsulinemic-euglycemic clamp approach (McGuire *et al.*, 1995; Griinari *et al.*, 1997b) have reported that both milk protein content and yield of cows with a 4-day clamp were significantly increased suggesting a potential stimulatory role of insulin on milk protein synthesis in dairy cows although the interpretation of the results is still open to debate since it is confounded by differences between treatments in ME supply.

1.5.1.2. Glucagon

As in other species, glucagon, a 29 amino acid polypeptide secreted by α-cells of the islets of Langerhans, is a potent hyperglycaemic hormone in ruminants. Glucagon dominates the regulation of hepatic metabolism while insulin may dominate the regulation of metabolism by peripheral tissues. Glucagon is also known to antagonise some actions of insulin and the serum insulin:glucagon ratio is thought to be important in the control of liver metabolism (Basset, 1975).

The major action of glucagon is acceleration of both hepatic glycogenolysis and gluconeogenesis resulting in stimulation of hepatic glucose output. A study with multi-cannulated, fed sheep (Brockman and Bergman, 1975) has shown that the net hepatic uptake of alanine and glutamine, and the conversion of alanine to glucose were increased by portal infusion of glucagon. It was also shown that arterial plasma concentrations of alanine and glutamine were decreased by 25 % during glucagon administration, presumably as a result of their increased hepatic uptakes. In a consecutive study by the same research group, glucagon directly stimulated the hepatic uptake of amino acids (i.e.

alanine, glycine, glutamine, arginine, asparagine, threonine, serine) and lactate in fed sheep (Brockman *et al.*, 1975). However, an *in vitro* study with an ovine liver perfusion technique found that glucagon caused a 76 % decrease of [^{14}C] threonine utilisation by ovine livers, and $^{14}CO_2$ production from [^{14}C] threonine was only 38 % of control when glucagon was infused suggesting that glucagon may mediate amino acid sparing by ruminant liver (Gill *et al.*, 1985).

The effect of glucagon on propionate utilisation in gluconeogenesis in the ruminant has also been studied both *in vivo* and *in vitro*. Glucagon administration did not alter glucose production from [2-^{14}C] propionate in sheep *in vivo* (Brockman and Greer, 1980) whereas glucagon stimulated glucose synthesis from propionate in the perfused ovine liver (Gill *et al.*, 1985). The stimulatory effect of glucagon on gluconeogenesis in ruminant lamb hepatocytes was smaller for propionate that for lactate or alanine (Savan *et al.*, 1986). Although it is difficult to reach a firm conclusion from the very limited information, glucagon seems to play an important role in maintaining glucose output when amino acids are the major glucose precursors, but has quantitatively less, if any, regulatory role in gluconeogenesis from propionate in ruminants judging by the concentrations required to stimulate glucose production from propionate in the *in vitro* studies.

It was proposed that glucagon may play an important role in supplying milk precursors to the mammary gland and may thus influence milk production (Brockman, 1978). Indeed, higher milk production throughout lactation was associated with higher glucagon concentration in lactating dairy cows, although glucagon concentrations were similar throughout lactation (Herbein *et al.*, 1985; Sartin *et al.*, 1985). Interestingly, studies with positive milk production responses to casein infused post-ruminally in lactating dairy cows have also reported increased glucagon concentrations in blood suggesting possible involvement of glucagon in milk production in response to protein supplementation (Cohick *et al.*, 1986; Choung and Chamberlain, 1995a).

1.5.1.3. Growth hormone, IGF-I and the somatostatin axis

Growth hormone (GH), a single polypeptide chain hormone of about 200 amino acid residues with two disulphide bridges, is produced in the anterior pituitary and comprises up to 10 % of the weight of a dried pituitary, far in excess of any other hormone present (Lewis, 1992). Its release in ruminants, as in most mammals, is pulsatile and occurs episodically. There are wide variations in hormonal release patterns between individuals in ruminants as in non-ruminant animals and they are probably determined, in part, genetically (Wallis *et al.*, 1985).

Feeding causes a marked decrease in plasma concentration of GH and the concentration increases while the animal is deprived of food (figure 1-5). In sheep, GH concentrations were found to be negatively correlated with the digestible organic matter intake (Bassett *et al.*, 1971). It appears that the effects of feed intake are related to energy balance of the animal rather than to feed intake per se. Indeed, marked changes in plasma concentrations of GH throughout the lactation cycle have been observed in sheep (Vernon *et al.*, 1981) and dairy cows (Hart *et al.*, 1978).

Growth hormone acts directly on target tissues when receptors are present. The adipocyte and the hepatocyte are well established as major direct targets of the hormone. However many of its actions are indirect and are mediated via insulin-like growth factors, IGF-I and IGF-II, which are released mainly by the liver and possibly by most body tissues. IGF-I and IGF-II are key members of the somatomedins, a group of related polypeptide hormones involved in promotion of cell proliferation and / or DNA synthesis, and stimulation of both protein synthesis and glucose uptake and metabolism (Hall and Sara, 1983). In all species studied, IGF-I is the predominant somatomedin after birth while IGF-II apparently dominates before birth (Butler and Gluckman, 1986). Effects of GH on

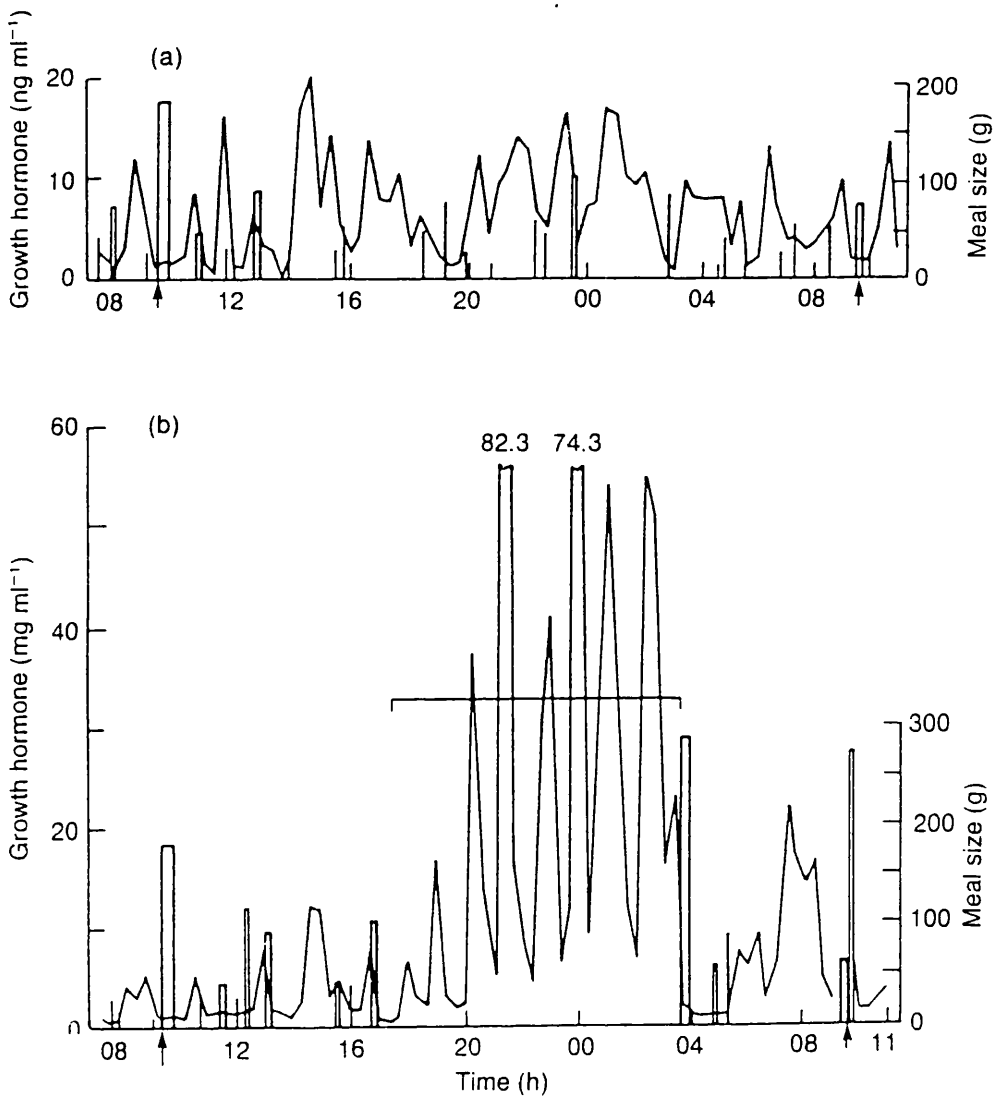


Figure 1-5. Plasma growth hormone concentrations and meal sizes during 27 h periods in a sheep: (a) with free access to food throughout; (b) food removed from 17:30 to 03:30 h (After Driver and Forbes, 1981)

mammary tissues are thought to be indirect since mammary epithelial cells lack GH receptors.

Commercial interest in the use of recombinant bST (bovine somatotrophin), exogenous growth hormone, to improve efficiency of animal production has led to extensive research efforts in this area for the past two decades and there are a number of reviews available on its effects on the metabolism of producing animals (Johnsson and Hart, 1986; Peel and Bauman, 1987; Bauman and Vernon, 1993). Briefly, when bST is administered milk yields can be increased substantially and significantly and, in general, increases of between 10 and 15 % are commonly obtained in ruminants (Bines and Hart, 1982). It also causes increased hepatic gluconeogenesis in dairy cows *in vivo* (Cohick *et al.*, 1989) and *in vitro* (Pocius and Herbein, 1986; Knapp *et al.*, 1992) possibly via a decreased ability of insulin to inhibit gluconeogenesis (Boisclair *et al.*, 1989). Glucose clearance after an intravenous insulin challenge is also decreased in bST-treated ruminants representing changes in hepatic and / or peripheral tissue metabolism (Hart *et al.*, 1984; Sechen *et al.*, 1990).

Therefore the galactopoietic actions of GH are both direct and indirect and indirect actions are apparently mediated by the IGF-I system. Administration of somatotrophin to dairy cows increases the plasma levels of IGF-I (Vicini *et al.*, 1991) and intra-arterial infusion of IGF-I into the mammary gland of the goat increases milk secretion and mammary blood flow (Prosser *et al.*, 1990) although it has also been reported that a three-day jugular infusion of IGF-I had no effect on milk yield (Davis *et al.*, 1989). The differences in response to IGF-I *in vivo* could arise from problems relating to IGF-binding proteins (see below). The high concentrations of GH during lactation observed in many studies (Hart *et al.*, 1978; Tindal *et al.*, 1978; Falconer *et al.*, 1979; Vicini *et al.*, 1991) are in line with the galactopoietic actions of GH. However, plasma levels of IGF-I decrease in early lactation. A study of IGF-I concentrations in cows throughout pregnancy and

lactation (Kerr *et al.*, 1991) found that the concentrations dropped by 50 to 100 % at the start of lactation. Similar findings were made in goats (Nielsen *et al.*, 1990). Furthermore, IGF-I is a growth factor stimulating anabolic processes in muscle and other body tissues and thus might be expected to have a negative effect on nutrient and energy availability to mammary tissues, raising the question of how GH through IGF-I can stimulate milk formation in the mammary gland.

Since their discovery in 1988, research focus has been directed toward IGF-binding proteins (IGFBPs) in relation to the actions of IGFs. IGFBPs are a family of six secreted proteins that bind to IGF-I and -II with high specificity. IGF-I circulates in plasma bound to different IGFBPs and the affinity constants of the IGFBPs are between two- and fifty-fold greater than the IGF-I receptors, hence effectively controlling the distribution of the IGF-I (Jones and Clemmons, 1995). The best known IGFBPs are IGFBP 1, 2 and 3, and IGFBP 1 and 2 are found in the 40 to 50 kDa molecular complex whereas the 150 kDa complex contains IGFBP 3.

In plasma of sheep, only 8 to 10 % of the total IGF-I in the plasma was found in the free form, 30 to 50 % was in the 40 to 50 kDa complex and 40 to 65 % in the 150 kDa complex (Hodgkinson *et al.*, 1991). It was also found in the same study that treatment with GH increased IGF-I level in both high and low nutritional status in sheep (although the effect was greater in the group with high nutritional status) and increased levels of IGF-I were associated with increased proportions of total IGF-I in IGFBP 3 bound form. *In vivo* studies with lactating cows (Vicini *et al.*, 1991; Cohick *et al.*, 1992) found that bST treatment resulted in not only an increase in IGF-I level but also a threefold elevation of circulating IGFBP-3 and a decrease in circulating concentrations of IGFBP 2.

The functions of IGFBPs are not well established yet. It is however apparent that IGFBPs act not only as carrier proteins, but also as modulators of IGF actions by participating in IGF ligand-receptor interactions through influences on both the

bioavailability and distribution of IGFs in the extracellular environment (Nielsen and Riis, 1993). Further knowledge of the role of IGFBPs and the significance of changes in their pattern of distribution (e.g. proportion of bound IGFs to each IGFBP to total IGFs) in relation to rate of milk formation and metabolic status in non-mammary body tissues may give answers to the question of how galactopoietic actions of GH through IGF-I occur.

The hypothalamus produces two neurohormones affecting GH secretion. They are growth hormone-releasing hormone (GHRH) and somatostatin. GHRH is a peptide of about 44 amino acid residues which specifically stimulates the secretion of GH by increasing cAMP production (Kelly, 1990). Lactating rats immunised with antiserum raised against GHRH had no increase in GH concentrations in plasma whereas other lactating rats with normal rabbit serum showed significant increases, indicating that GHRH is directly responsible for the increase in GH during lactation (Wehrenberg and Gaillard, 1989). Somatostatin, originally known as SRIF (somatotrophin-release inhibiting factor), on the other hand specifically inhibits pituitary GH secretion (Kelly, 1990). Somatostatin can be secreted both locally and into the general circulation, thus exerting both paracrine and endocrine effects. There are two principal biologically active polypeptides present in plasma which originate from cleavage of the prosomatostatin molecule. These are somatostatin-14 (SS-14) and somatostatin-28 (SS-28). It is believed that SS-14 is produced mainly in the pancreatic and gastric cells whereas SS-28 is synthesised in the proximal intestinal epithelial cells (Ensinck *et al.*, 1989); the somatostatin-like immunoreactivity (SLI) is known to be present in virtually every tissue in the body in most organisms (Yamada and Chiba, 1989). The distribution of the two molecular forms of somatostatin is different among tissues but its physiological significance is not clear (Chiba and Yamada, 1994). Apart from inhibition of GH release, both SS-14 and SS-28 are known to affect a variety of physiological functions in simple-stomached animals including inhibition of gut motility and gastric emptying, and inhibition of secretion of pancreatic hormones (i.e.

insulin, glucagon and pancreatic polypeptide) and polypeptides (i.e. CCK, gastrin, gut glucagon, GIP, GLP-1, motilin, secretin and VIP) secreted by the small intestine (Patel, 1990; Chiba and Yamada, 1994). Much less is known in ruminant animals but the position is thought to be similar. Indeed, exogenous administration of SS-14 significantly reduced the plasma concentration of GH and insulin in sheep (Rose *et al.*, 1996). Similarly, intravenous SS-28 infusion in sheep also suppressed circulating concentrations of GIP and GLP-1 as well as serum insulin levels induced by glucose and GLP-1 (Martin and Faulkner, 1996) and by butyrate (Holtenius and Hydbring, 1993). The inhibitory effect by somatostatin is believed to occur in part via antagonising the rise in cAMP through interaction with G_i protein (Fehmann *et al.*, 1993). However, its physiological role in regulation of nutrient partitioning via controlling pancreatic and gut hormone release, particularly in lactation, is yet to be elucidated.

1.5.2. Gut hormones and the entero-insular axis

Traditionally, gut hormones (or gastrointestinal hormones) have been defined as peptides produced by endocrine cells located in the gastrointestinal mucosa, released into the circulation under the influence of alimentary stimuli, which are involved in the regulation of secretion, motility and growth in the digestive system. However, it is now recognised that a number of gut peptides are also produced by neurons in the central and peripheral nervous system, particularly enteric neurons (Desbuquois, 1990).

There are more than a dozen gut hormones found in the gastrointestinal tract of humans including gastrin, secretin, cholecystokinin (CCK), glucose-dependent insulinotrophic polypeptide (GIP), glucagon-like peptides (GLP-1 and GLP-2), pancreatic polypeptide (PP), motilin, vasoactive intestinal peptide (VIP), somatostatin, neurotensin, bombesin, peptide YY (PYY), substance P and opioid peptides (Desbuquois, 1990;

Creutzfeldt and Nauck, 1992; McIntosh, 1995). In ruminant animals, there is very limited information available although existence of the peptides and basic mechanisms are expected to be similar.

Many gut hormones are known to be involved in the regulation of major metabolic hormones especially pancreatic hormones (e.g. insulin). Indeed, the concepts of 'incretin' and 'entero-insular axis' originate from the observations that a component of small intestinal extracts augmented pancreatic endocrine responses and lowered plasma glucose (Moore *et al.*, 1906; Zunz and La Barre, 1929), and that greater amounts of glucose could be given orally, rather than intravenously, without causing glucosurea (Unger and Eisentraut, 1969; Morgan, 1992).

Two gut hormones, generally recognised as being major components of the entero-insular axis with most potent insulin-stimulating activity, are GIP and GLP-1.

GIP, also known as 'gastric inhibitory polypeptide' since it was originally characterised in terms of its gastric acid inhibitory properties, is a 42-amino acid polypeptide secreted by intestinal K cells principally in the duodenum and proximal small intestine (Solcia *et al.*, 1974; Cleator and Gourlay, 1975). In simple-stomached animals, GIP is released in response to nutrient absorption. It has long been established that oral glucose stimulates GIP in simple-stomached animals (Cataland *et al.*, 1974; Kuzio *et al.*, 1974), and the glucose-induced GIP secretion is dose-dependent (Martin *et al.*, 1975; Schulz *et al.*, 1981). Fat is a particularly potent GIP secretagogue (Brown, 1974; Falko *et al.*, 1975) and GIP secretion in response to triacylglycerol ingestion is dependent upon fatty acid absorption. Thus, cholestyramine, which impairs micelle formation and decreases fat absorption, leads to reduced GIP secretion (Ebert and Creutzfeldt, 1983). Notably, long-chain fatty acids (C18) seem to be more potent in inducing GIP secretion compared with short- and medium-chain fatty acids in simple-stomached animals (Ross and Shaffer, 1981; Kwasowski *et al.*, 1985). Although the reasons for this are not clear, it

was proposed that esterification within the enterocyte may be a prerequisite for GIP secretion since long-chain fatty acids are esterified before incorporation into chylomicrons and secretion into the general circulation, whereas short- and medium-chain fatty acids are transferred across the intestinal cells without esterification and enter the portal vein as free fatty acids (Clement, 1980; McCarthy, 1993). Intraduodenal administration of a mixture of free amino acids also stimulated GIP secretion, although to a lesser degree than fat and carbohydrate (Thomas *et al.*, 1976). However, protein ingestion does not elicit GIP secretion when given as a meat extract (Brown, 1974), fillet steak (Cleator and Gourlay, 1975), or turkey steak (Elliot *et al.*, 1993).

Once secreted into the circulation, GIP is known to act in at least two ways in the regulation of nutrient utilisation, first by augmenting insulin secretion in response to nutrient absorption and secondly, by direct anabolic effects in adipose tissue (Brown *et al*, 1989; Pederson, 1994). GIP was the first incretin in potentiating insulin secretion when given intravenously, but its insulinotrophic effect is dependent on glucose concentration. Elahi *et al.* (1979) reported that the glucose concentration threshold for the insulinotrophic effect in humans is 5.5 mM, approximately 1.4 mM above the basal level, below which GIP will not stimulate insulin release. Thus, fat-stimulated GIP secretion will not influence insulin secretion unless hyperglycaemia is achieved, for example after ingestion of a mixed meal or by intravenous glucose infusion (Cleator and Gourlay, 1975; Crockett *et al.*, 1976).

In addition to effects on the pancreas, GIP has direct actions on adipose tissue metabolism. Physiological concentrations of porcine GIP (0.2 to 4 ng / ml) have been shown to stimulate fatty acid synthesis in rat adipose tissue (Oben *et al.*, 1991). The uptake and incorporation of glucose into extractable lipid in rat adipocytes were also enhanced by GIP although the concentrations applied were unphysiological (Hauner *et al.*, 1988).

GIP is also present in ruminant animals having been isolated from bovine small intestine (Carlquist *et al.*, 1984). It is secreted in response to feed intake in sheep

(McCarthy *et al.*, 1992). However, the primary stimulus for GIP secretion in ruminants appears to be fat rather than glucose as feeding milk but not glucose or lactose evokes GIP secretion in goats (Martin *et al.*, 1993) and lambs (Martin and Faulkner, 1994), and glucose is not a stimulus for GIP secretion in anaesthetised goat kids (McCarthy *et al.*, 1991).

In contrast to simple stomached animals, GIP does not appear to be insulinotrophic in ruminants. In sheep, intravenous GIP administration had no effect on insulin concentration and furthermore increases in insulin concentration when glucose was injected intravenously were unaffected by concurrent administration of GIP (Martin and Faulkner, 1993). A similar inability of GIP to promote insulin secretion has also been reported in adult sheep (Faulkner, 1990). However, further confirmation would be necessary before concluding that GIP is not insulinotrophic in other ruminant animals.

Despite its lack of insulinotrophic effects in the ruminant, GIP does appear to act as an anabolic agent in adipocytes in the ruminant. A study with a microdialysis technique *in vivo* has shown that intravenous GIP infusions reduce concentrations of glucose in the perfusates of ovine adipose tissue through direct stimulation of lipogenesis (Martin *et al.*, 1993). Furthermore, Haji Baba and Buttery (1991) reported a strong positive effect of GIP on acetate incorporation in ovine adipose tissue *in vitro*.

GLP-1 is another incretin that is capable of stimulating insulin secretion. It was initially characterised through its ability to cross-react with antisera raised against pancreatic glucagon and was originally designated as one of glucagon-like immunoreactants or GLIs (see Morgan, 1992). Its precursor, proglucagon, which is synthesised in both the pancreas and the gut, is a 160-residue peptide that is processed differently in the pancreas and small intestine (Mojsov *et al.*, 1986). GLP-1 is cleaved from proglucagon processed in the gut alongside GLP-2, glicentin and oxyntomodulin which is further cleaved to GLP-$1_{(7\text{-}36)}$ amide (Morgan, 1992). It is this truncated form of GLP-1 that is the major circulating form following a meal in man (Ørskov *et al.*, 1987).

Like GIP, GLP-1 is not a potent stimulant of insulin secretion at normal blood glucose levels and becomes stimulatory during periods of hyperglycaemia (Goke *et al.*, 1991; Ørskov, 1992). It has been found that GLP-1 possesses a potency equivalent to (Holst *et al.*, 1987) or greater than (Shima *et al.*, 1988) GIP in stimulating insulin secretion on a molar basis from the isolated pancreas in simple-stomached animals although its circulating concentrations were found to be lower than those of GIP in humans (Kreymann *et al.*, 1987; Takahashi *et al.*, 1990). In ruminant animals, GLP-1, in contrast to GIP, seems to be insulinotrophic. Giving intravenous GLP-1 to starved sheep increased the insulin response to the intravenous glucose load (Faulkner and Pollock, 1990; Martin and Faulkner, 1993). However, GLP-1 on the other hand did not augment insulin secretion in response to other known insulin secretagogues such as propionate, octanoate and arginine (Martin and Faulkner, 1993).

GLP-1 also seems to share with GIP the ability to stimulate *de novo* fatty acid synthesis in adipose tissue (Oben *et al.*, 1989). However, a lack of information together with the fact that the L cells which secrete GLP-1 are located at the distal region of the small intestine make it difficult at present to access its physiological function in lipid metabolism in both ruminant and non-ruminant animals.

1.6. AIMS AND OBJECTIVES

The differences in responses of milk production to abomasal infusions of casein, casein hydrolysates and corresponding mixtures of free amino acids are difficult to explain in terms solely of amino acid supply. Other factors appear to be involved, one of these being the possible generation of bioactive peptides during digestion. The objective of the work described in this thesis was to investigate the effects of casomorphins on the metabolism of the cow. Essentially, the experiments described fall into three groups.

1) At the start of the project, it was recognised that casomorphins could exert their effects on the gut itself or, following entry into the bloodstream, on tissues beyond the gut. Evidence already pointed to effects of casomorphins on amino acid uptake from the gut and this raised the possibility that absorbed casomorphins might similarly influence amino acid uptake by body tissues, especially the mammary gland. This possibility was examined first and the absence of detectable effects on the rat mammary gland *in vitro* led to the decision to focus the rest of the work on effects at a gut level.

2) With regard to the route of administration of casomorphins to be used in the experiments, it was important to determine whether addition of the peptides to the diet could deliver them effectively to the small intestine. Hence, the stability of the casomorphins in the rumen was measured and a preliminary investigation made of the feasibility of protecting the peptides from ruminal degradation.

3) The main part of the thesis dealt with the effects on blood levels of hormones and metabolites when casein and its digestion products, including casomorphins, were infused direct into the abomasum of the cow.

CHAPTER TWO

MATERIALS AND METHODS

Materials and methods associated with *in vivo* experiments and hormone and metabolite analysis are described here. Materials and methods specifically associated with the studies of Chapter 4, 5 and 6 are described in their respective chapters.

2.1. PREPARATION OF SURGICALLY AND EXPERIMENTALLY MODIFIED ANIMALS

2.1.1. Rumen cannulation

The animal was fasted and denied access to water for 24 hours prior to the operation. Hair was removed from the area of the operation by clipping and shaving. Immediately prior to the operation the animal was weighed and anaesthesia was introduced into vertebrae of the animal by injection of local anaesthetic (Lignavet, C-Vet Veterinary Products, Leyland, Lancs., UK).

The operation was performed with the cow in a standing position. The area of operation was scrubbed with an antiseptic solution. For the insertion of the cannula, a 12 cm incision was made with a scalpel below the transverse processes of the lumbar vertebrae and 10 cm posterior to the last rib of the cow. The muscle layers of the abdominal wall were separated along the direction of travel of their muscle fibres and retracted to expose the peritoneum, which was incised to expose the rumen wall. A pouch of ventral rumen wall was exteriorised and clamped completely. The rumen wall, attached layers and skin were stitched together paying special attention to stitching securely at the lower end of the incision. The exteriorised rumen was wrapped with swabs and tape. Ten days later, the clamp and the exteriorised rumen were removed. A bung made from cotton

wool, in a plastic bag, was inserted into the hole and secured with elastic bandage over the area. The bung was left in position for 3 days or, if necessary, until the fistula was large enough to allow insertion of a rumen cannula with rolled inner flange (Bar Diamond Lane, Parma, Idaho, USA). The flange of the cannula was introduced into the rumen, the stem was exteriorised through the body wall, and the rubber stopper was inserted into the cannula.

2.1.2. Insertion of abomasal infusion catheter

Each animal was fitted with an infusion catheter into the abomasum at least a day before the start of the experiment. A large rumen cannula allowed the positioning by hand of an infusion line into the abomasum, secured in position by a plastic bottle and rubber cuff (Derrig et al., 1974). The pH of withdrawn digesta was routinely checked twice daily to ensure that the catheter was positioned in the abomasum and the cannula and the infusion tubing were flushed with warm water every morning prior to the start of infusion.

2.1.3. Jugular catheterisation

The site of venipuncture on the neck of the animal was sterilised with Hibitane solution containing 10 % (v/v) Hibitane (5 %, v/v; Zeneca Ltd., Macclesfield, Cheshire, UK), 75 % (v/v) ethanol and 15 % (v/v) distilled water. All catheterisation was completed about 18 hours before the start of infusion and blood sampling. Intravenous catheterisation was performed in two ways. When an intravenous catheter was required only for experiments where less frequent (at least 30 minute intervals) blood sampling was required at the end of a period of treatments lasting at least a week, a temporary catheter was inserted as follows. Using a Medicut (14g; Sherwood Medical, Tullamore, Ireland) one of the jugular veins was punctured and a Single Lumen Polyethylene Tube (ID 1.0 x OD 1.5 mm; Silverwater BC, N.S.W. 2128, Australia) was inserted in to the lumen of the jugular

vein through the Medicut until approximately 20 cm lay in the vein. An adhesive bandage (5 cm, Treatplast; Animalcare, Dunnington, York, UK) was used to cover and secure the catheter. The catheter was removed at the end of each day of blood sampling. When an intravenous catheter was required for experiments with shorter periods (3 to 4 days) involving an intravenous infusion with more frequent (15 minutes) blood sampling, the following method was used. One of the jugular veins was punctured with a Medicut and a stainless steel guide wire (Meadox Ltd., Dunstable, Beds., UK) was inserted instead of the polyethylene tube as described before. An indwelling polyethylene catheter (14g x 8 cm; Arrow International Inc., Reading, Berks., UK) was inserted into the lumen, being guided by the wire and the catheter clamp was connected with a tubing line (200 cm; Kimal Scientific Products Ltd, Uxbridge, Middlessex, UK) and attached to the skin using superglue. The adhesive bandage was also used to cover and secure the catheter. The bandage was changed and the catheter was checked and corrected when required on the day before a blood sampling day throughout the whole experiment. Patency of the catheter was maintained by flushing it with a sterilised sodium citrate saline solution of 0.9 % (w/v) sodium chloride and 0.5 % (w/v) trisodium citrate every morning before the start of blood sampling.

2.2. EXPERIMENTAL TECHNIQUES

2.2.1. Intravenous infusion

Only one jugular vein was catheterised for intravenous infusions and blood sampling to minimise stress on the cow caused by the operation and the maintenance of catheterisation.

A bolus injection of glucose at concentrations of 36 or 72 mg / kg BW (body weight) was given manually with a 50 ml syringe. The injection was completed within 1

minute. For continuous intravenous infusions of glucose, solutions of glucose (1 or 2 mg / kg BW / min) were infused into the jugular vein using a 50 ml syringe and a syringe pump for 10 minutes at a rate of 2.5 ml / min with intervals of 15 minutes. This allowed 5 minutes for blood sampling through the same jugular catheter. The infusion line was washed by flushing it with 15 ml of the saline solution before and after each blood sampling. Intravenous infusion of somatostatin-28 was given by the same method as for the continuous glucose infusion at a rate of 52 pg / kg BW / min for 15 min.

2.2.2. Intra-abomasal infusion

A solution of glucose in a volume of 1 litre water was infused into the abomasum using a single-channel peristaltic pump (Watson and Marlow 502S; Watson and Marlow Ltd, Falmouth, Cornwall, UK) with 1.6mm ID silicon tubing (Belmont Instruments, Glasgow, UK) at a rate of 4 l / h. A bolus infusion of a mixture of β-casomorphins in 20 ml water was given using a 25 ml syringe through the infusion line with a 20 ml flush of water before and after the infusion.

2.2.3. Preparation of infusates

For abomasal infusion, distilled water was used to dissolve β-casomorphins or glucose. Solutions of glucose for the intravenous infusion were prepared by the gradual addition of glucose to distilled water kept warm at about 60 °C with constant stirring. The solutions were then filtered through a cellulose-nitrate membrane filter (pore size in 0.45 μm; Whatman Ltd., Maidstone, UK) and sterilised at 121 °C for 15 minutes.

2.3. COLLECTION AND PREPARATION OF SAMPLES

2.3.1. Feedstuffs

Silage samples were taken from the silo once before the start of each experiment and once during each experiment, and a subsample of 1500 g was dried and ground through a 1 mm screen before analysis. Another subsample of 1500 g was minced through a 100 mm dye (Crypto Ltd., London, UK) and stored frozen for further analysis.

All other feedstuffs were sampled at least once during each treatment period. Subsamples were dried in triplicate at 60 °C in a forced-draught oven for dry matter determination and ground through a 1 mm screen and stored for analysis.

2.3.2. Milk

Milk samples were collected from the afternoon milking on the day on which the experimental treatments were given and from the morning milking on the next day. Approximately 300 ml of milk were collected into bottles containing 180 mg potassium dichromate (Thompson and Capper Ltd, Runcorn, Cheshire, UK), mixed thoroughly to dissolve the preservative and stored at 4 °C until analysis. Successive samples from individual cows were then bulked in proportion to milk yield.

2.3.3. Blood

Samples of blood were withdrawn from a jugular vein via the intravenous catheter inserted prior to the start of the infusion. Blood samples were taken into 20 ml syringes and transferred into 15 ml heparinised tubes which were prepared by distribution of 143 unit heparin / 100 μl and dried at 60 °C. Samples were centrifuged at 1500 g for 15 minutes and the blood plasma was removed shortly after collection. Plasma was either snap frozen by transferring to a container containing liquid nitrogen or harvested and stored immediately

at -20 °C prior to analysis.

2.4. ANALYTICAL METHODS

2.4.1. Dry matter and ash

Dry matter and ash contents in samples of feedstuffs were determined by standard methods (Agricultural Development and Advisory Service, 1981) with the exception of silage, which was determined by distillation of a minced silage sample with toluene following the procedure of Dewar and McDonald (1961).

2.4.2. pH of silage

A representative samples of 20 g of wet silage was taken and mixed with 20 ml of distilled water and the pH was read using a pH meter (Hanna instruments, Leighton Buzzard, Beds., UK).

2.4.3. Total nitrogen

The N content of feed samples was measured by a macro-Kjeldahl method using a Kjeltec Auto 1030 analyser (Foss UK Ltd., Didcot, Oxon, UK).

2.4.4. True protein and non-protein nitrogen (NPN) in silage

The true protein content of silage was determined by Kjeldahl analysis of the material precipitated by tannic acid (Van Roth, 1939). NPN content was calculated by subtracting the true protein content from the crude protein content.

2.4.5. Ammonia nitrogen in silage

A water extract of the silage sample was prepared by placing 20 g wet minced silage and 200 ml distilled water in a beaker in a water bath at 40 °C for 30 minutes, stirring intermittently. The extract was then filtered by squeezing the silage juice through muslin, and was centrifuged at 1500 g for 20 minutes. Ammonia nitrogen in silage was determined by distillation of ammonia after addition of NaOH to the extract. Ammonia was collected in 0.02 M HCl and determined by titration.

2.4.6. Lactic acid in silage

Lactic acid was determined on a water extract by the method of Elsden and Gibson (1954) in which lactic acid is oxidised to acetaldehyde, which combines with sodium metabisulphate and is determined iodimetrically. Sugars, which may give rise to carbonyl compounds, and nitrogenous compounds such as protein are removed with copper sulphate and calcium hydroxide.

2.4.7. Total soluble sugars in silage

Total soluble sugars in silage were determined on a water extract by a method similar to that of Somogyi (1945). The calorimetric determination of sugars involved the use of a Somogyi reagent and an arsenomolybdate reagent.

2.4.8. Ethanol in silage

Ethanol was determined by gas chromatography by the method of Huida (1982) using methanol as an internal standard. Briefly, 30 ml of dry methanol were added to 5 ml of silage extract and 1 µl injected on to the column of a Shimadzu GC-8A gas chromatograph (Shimadzu Europe Ltd., Milton Keynes, Bucks., UK) fitted with a flame ionisation detector. The columns were 2 m long and of 2 mm internal diameter and were

packed with Chromosorb 101. The oven setting was 100 °C and the carrier gas (N_2) flow rate was 60 ml / min.

2.4.9. Total and individual volatile fatty acids (VFA)

The VFA in the silage were determined by gas chromatography by the procedure of Cottyn and Boucque (1968). The apparatus used was the Shimadzu GC-8A gas chromatograph with a glass column packed with 5 % Carbowax 20M / TPA on Chromosorb G 80 / 100 mesh. The oven temperature was 100 – 120 °C and carrier gas (N_2) flow rate was 60 ml / min. The molar concentration was calculated for each acid from the peak area on the chromatograph relative to that of hexanoic acid. Corrections were made for the differences in the response of the detector to each acid using factors derived from the analysis of a standard VFA solution.

2.4.10. Neutral detergent fibre (NDF) and acid detergent fibre (ADF)

The NDF and ADF contents in feed were determined by the standard methods of Goering and Van Soest (1970).

2.4.11. Milk fat

Milk fat was determined by the Gerber method according to British Standard 696 (1969). Fat was separated from the milk by the addition of concentrated sulphuric acid and measured directly using a Gerber butyrometer.

2.4.12. Milk protein

Total nitrogen in milk samples was determined by a macro-Kjeldahl method (Association of Official Analytical Chemists, 1975) using the Kjeltec Auto 1030 analyser. The N content was multiplied by 6.38 to obtain the concentration of crude protein.

2.4.13. Milk lactose

Milk lactose was determined using an automatic polarimeter (Thorn Automation Ltd., Nottingham, UK) according to the method of Grimbleby (1956).

2.5. HORMONE AND METABOLITE ANALYSIS

2.5.1. Preparation of radiolabelled insulin

The iodination procedure used was based on a method first described by Fraker and Speck (1978), in which ^{125}Iodine was incorporated into protein using the Iodogen™ reagent (Pierce Europe BV, Oud-Beijerland, Netherlands). Microtubes were coated with 30 µl of Iodogen reagent (50 µg/ml in chloroform) evaporated to dryness at room temperature and stored at -20 °C. The iodination was performed by adding 500 µCi (5 µl) ^{125}I-sodium iodide (IMS. 30, Amersham International, Amersham, Bucks., UK) to a 5 µg (5 µl) aliquot of insulin in an Iodogen-coated microtube with 10 µl phosphate buffer (0.4 M, pH 7.4). After incubating for 20 minutes, the reaction products were diluted with 100 µl of phosphate buffer and transferred to a column (1 x 15 cm; Biorad, Hemel Hempstead, UK) of sephadex G10 (Sigma Ltd.) which had previously been equilibrated with RIA buffer. To stop the reaction, the empty tube was washed with 200 µl of potassium iodide (2 %, w/v) which was then added to the column. RIA buffer was added carefully into the top of the column and fractions were collected every 2 to 4 minutes, and the radioactivity of each fraction was counted using a Geiger counter (Mini-instruments Ltd., Burnham-on-Crouch, Essex, UK) at a distance of 40 cm. Iodinated insulin eluted in the first peak and free iodine in the second peak (figure 2-1). The fractions with the highest level of radioactivity were combined and stored at -20 °C with 100 µl aliquots. Incorporation of ^{125}I into the hormone was determined by counting a small aliquot before and after precipitation of the protein

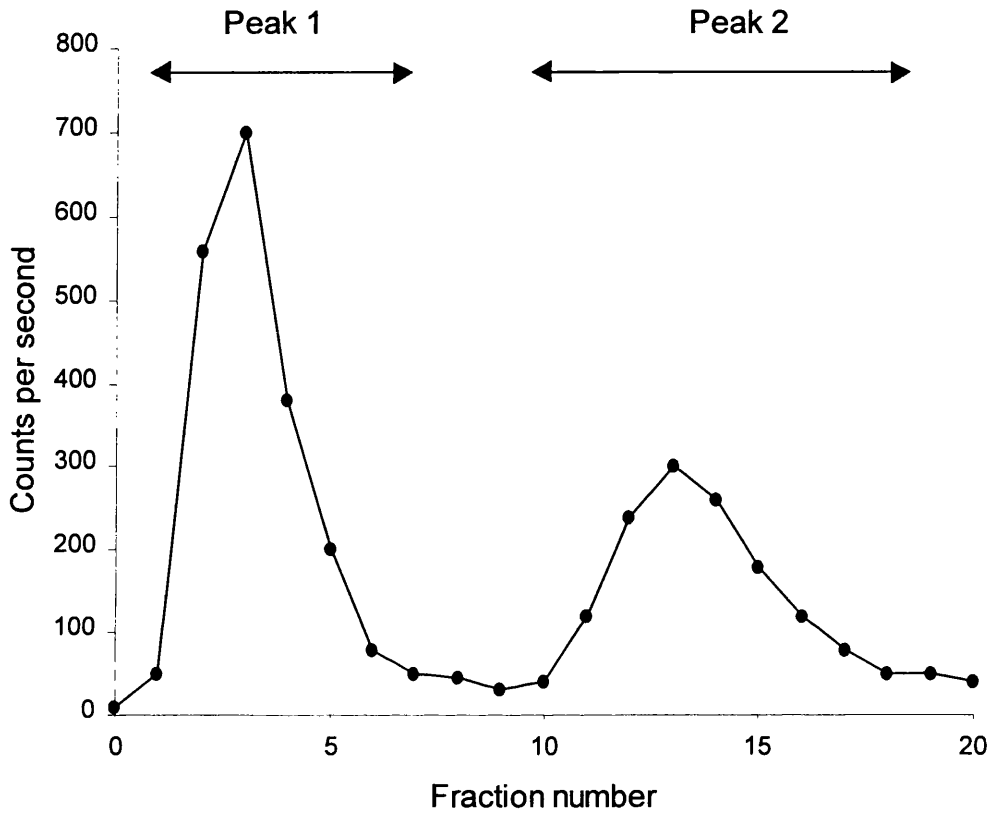


Figure 2-1. Separation of iodinated insulin from unbound iodine on a sephadex G10 column. ^{125}I-Insulin was eluted in the first peak and free iodine in the second peak. Fractions 3 to 5, containing precipitable ^{125}I-insulin, were combined and used as radiolabel in the insulin radioimmunoassay.

with 10 % (w/v) TCA. The specific activity of ^{125}I-insulin was calculated using equation 2-3:

$$\text{Specific activity } (\mu Ci / \mu g) = \frac{(B+D)}{(B+D+E)} \times \frac{A}{1500} \times \frac{500}{C} \qquad (2\text{-}3)$$

where A is ^{125}I added to insulin (cps at 60 cm; where 500 μCi = 1550 cps), B is residual ^{125}I not transferred to the column (cps at 60 cm; assumed to be ^{125}I-insulin), C is the amount of insulin iodinated (μg), D is ^{125}I present in insulin peak, E is ^{125}I present in iodine peak.

2.5.2. Insulin RIA (radioimmunoassay)

Reagent

RIA buffer:

0.05 M $Na_2PO_4 \cdot H_2O$, pH 7.4

0.15 M NaCl

0.02 % (w/v) sodium azide

0.5 % (w/v) BSA (bovine serum albumin)

Insulin standard:

Natural porcine insulin (Sigma Ltd.) was dissolved in 10 mM HCl then diluted in RIA buffer, batched in 10 μl aliquots (10 μg / ml) in eppendorf tubes and stored at -20 °C.

First antibody:

Antiserum to bovine insulin was donated by SAPU (Scottish Antibody Production Unit, Glasgow, UK). Final dilution of 1:40,000 was used in the assay.

Second antibody:

140 mg EDTA (ethylene diaminetetra-acetic acid) in 15 ml RIA buffer (adjusted to

pH 7.4 after adding EDTA), then 5 µl normal guinea pig serum and 125 µl anti-guinea pig precipitating serum (SAPU) and 15 ml of 16 % (w/v) PEG (polyethylene glycol).

Assay procedure

The insulin assay was based on the method described by Vernon et al. (1981). Insulin standards ranged from 0.05 to 10 ng / ml. A volume of 100 µl was taken, in duplicate, for standards and samples and 100 µl of first antibody was added to this to give a final antiserum dilution of 1:40,000. After incubation at room temperature for 6 hours, ^{125}I-insulin was added to all tubes (100 µl; 10,000 cpm). Tubes were incubated overnight (for at least 8 hours) before the addition of second antibody (300 µl). Tubes were incubated for a further 2 hours at room temperature, then centrifuged at 2,000 g for 30 minutes and the supernatant decanted. The protein precipitate, containing antibody-bound ^{125}I-insulin, was counted on a gamma counter (Cobra Auto-gamma, Packard, Pangbourne, Berks., UK). The concentration of insulin in the samples was determined by interpolation from a standard curve.

2.5.3. Glucagon RIA

Glucagon RIA was carried out using a Double Antibody Glucagon Kit (KGND1; Diagnostic Products Corporation, Los Angeles, CA 90045-5597, USA) and the procedure was the same as insulin RIA except 24 hours incubations at 2 to 8 °C after both first and second antibody additions.

2.5.4. GH RIA

Antiserum to ovine GH (AFP-CO123080, donated by NIH, National Institutes of Health) was used with a dilution of 1:20,000, and the standard curve (0.31-40 ng / ml) was

prepared from ovine GH (AFP-9220A). The procedure described for insulin was followed except that the addition of the tracer (20,000 cpm; ^{125}I-bST) was delayed 24 hours after the addition of the first antibody to standard curve and sample tubes.

2.5.5. GIP RIA

The GIP assay was based on the method described by Morgan et al. (1978). GIP standard ranged from 0.125 to 4 ng / ml and the RIA buffer used was 0.1 M phosphate, 0.05 M NaCl, 0.01 % sodium azide, 0.1 % triton and 0.1 % BSA. Natural porcine GIP and first antibody (rabbit anti-porcine GIP antiserum) were purchased from Guildhay Antisera Ltd. (Guildford, Surrey, UK). The rest of the reagents and chemicals were as used in the assay of insulin. The procedure itself was also as described for insulin RIA except that the volume of first antibody, tracer and second antibody were 50, 50 and 200 µl respectively.

2.5.6. GLP-1 RIA

GLP-1 was determined by RIA using an antibody raised in rabbits (Peninsula Laboratories Inc., St Helens, Merseyside, UK). ^{125}I-GLP-1 was prepared by coupling GLP-1 to Na ^{125}I (ICN, Thame, Oxon, UK) using iodogen. The assay was performed in 0.1 M phosphate buffer pH 7.4 containing 0.05 M NaCl, 0.01 % sodium azide and 0.5 % BSA by the same procedure as used for GIP RIA.

The antibody reacts specifically with the C-terminal end of the peptide and both (7-36) amide and (1-36) amide forms of the peptide are recognised. Similarly, the antibody will probably recognise the (9-36) amide which is produced in plasma as a result of dipeptidase activity (Mentlein et al., 1993; Deacon, et al., 1995).

2.5.7. IGF-1 RIA

The method described by Flint and Gardner (1989) was used for the determination

of IGF-I concentration in the plasma after the samples were extracted with acid-ethanol, to separate the IGF-I from its binding proteins. The extraction was done by adding 4 volumes of the extraction medium (2M HCl and ethanol 1:7, v/v) to 1 volume of the samples and the standard tubes and incubating for 30 minutes at room temperature. The tubes were centrifuged at 3000 g for 10 minutes; after that, a specific amount of supernatant was removed and an equal amount of neutralising buffer (4%, w/v, TRIS : RIA buffer) was added and then the samples were further diluted with RIA buffer. Recombinant human IGF-I (Bachem, Saffron Walden, Essex, UK) was used to construct the standard curve (10 – 2500 ng / ml). The first antibody, polyclonal rabbit anti-rhIGF-I (a gift from NIDDK, Bethesda, Maryland, USA), at a dilution of 1:2000 was added to standards and sample tubes and incubated for 24 hours before adding ^{125}I-IGF-I (approximately 20,000 cpm per tube) and then incubated overnight at room temperature. The second antibody (RIA buffer / 16 % PEG with equal volume and 6 % (v/v) anti-rabbit IgG precipitating serum and 0.4 % (v/v) normal rabbit serum, both from SAPU) was added to the tubes and further incubated for 2 to 4 hours before centrifugation at 3,000 g for 30 minutes at room temperature. The pellet was counted and IGF-I concentration was determined as described for insulin.

2.5.8. Plasma glucose assay

Plasma glucose concentrations were determined using a method described by Bergmeyer and Bernt (1974). Glucose was oxidised by the enzyme glucose oxidase to give hydrogen peroxide and glucuronic acid. The hydrogen peroxide then reacted with O-dianiside to yield a coloured product. The final colour intensity was proportional to the glucose concentration.

Reagent

Combined Enzyme-Colour Reagent RIA buffer:

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

1 % (w/v, in 95 % ethanol) O-dianisidine; 50 µl

Peroxidase, 40 units / ml

Glucose oxidase, 30 units / ml

Glucose standard, 1 mM

Procedure

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

CHAPTER THREE

EFFECT OF β-CASOMORPHINS ON AMINO ACID UPTAKE BY LACTATING RAT MAMMARY GLAND

3.1. INTRODUCTION

β-casomorphins have been found to be released during the course of digestion *in vivo* (Meisel *et al.*, 1989). They are proline-rich peptides with high resistance to hydrolysis by pancreatic proteases (Brantl and Teschemacher, 1979). This could increase the concentration of the peptides at sites of absorption in the intestine. Although their intact absorption via the active PEPT-1 transport system is highly unlikely, their accumulation at the sites of absorption together with an available route of absorption (i.e. paracellular pathways) could lead to systemic availability of the peptides.

One of the reported actions of β-casomorphin is a stimulation of amino acid uptake in the small intestine (Ermisch *et al.*, 1989). Since amino acid transport systems present at the enterocytes of the small intestine and the mammary gland are similar, it is possible that systemically available β-casomorphins might also directly stimulate amino acid uptake by the mammary gland.

The main objective of the present work was to determine the effect of β-casomorphins on the uptake of amino acids by the mammary gland. *In vitro* experiments were conducted, using mammary tissue explants taken from lactating rats during peak lactation. Four amino acids, L-glutamate, L-histidine, L-leucine and L-lysine, were selected as representative of amino acids using different transport systems. Preliminary experiments were also carried out to determine both the feasibility of the technique and the optimum experimental conditions for the main experiments.

3.2. MATERIALS AND METHODS

Amino acid uptake by isolated mammary tissue explants was measured according to the method of Shennan (1989), modified from Pocius and Baumrucker (1980).

3.2.1. Animals

Primiparous females of the Wistar strain (A. Tuck and Son, Rayleigh, Essex, UK) were fed ad libitum on standard rat chow (CRM Irradiated Diet, Labsure, Cambridge, UK) and allowed free access to water. They were housed at a constant temperature of 17 °C and under 12 hour light/dark cycles. Animals were housed in groups of up to three individuals on wood shavings from mating and then singly prior to parturition. At parturition, animals were given shredded paper as nesting material and the pup numbers were adjusted to 10 per mother, where possible. Experiments were conducted on females at 9 to 15 days of lactation unless stated otherwise.

3.2.2. Chemicals and radiochemicals

A scintillation cocktail, UltimaGold, was obtained from Packard Instrument B. V. (Pangbourne, Berks., UK). Bovine serum albumin was fraction V obtained from Sigma Ltd. (Poole, Dorset, UK). All radio-labelled compounds were obtained from Amersham International plc (Little Chalfont, Bucks., UK). All other chemicals used in the amino acid uptake experiment were obtained from Sigma Ltd.

3.2.3. Acquisition of mammary tissue

The rats were lightly anaesthetised with ether and killed by cervical dislocation. The abdominal mammary glands were removed immediately and placed in an oxygenated/ice cold buffer containing 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM

glucose, 10 mM Tris-MOPS pH 7.4, 1 mM aminooxyacetic acid and 5 μM of amino acid (glutamic acid, histidine, leucine and lysine). The tissue was gassed with 100 % O_2 for 5 to 10 minutes to remove labile blood and milk from the tissue.

3.2.4. Explant preparation

The mammary tissue was removed from the incubation buffer and as much excess connective tissue as possible was removed from the surface of the gland. The mammary tissue was cut into fine strips of less than 1 mm in diameter. These strips were then finely dissected to produce explants, each weighing between 1 and 5 mg. The explants were returned to buffer on ice and gassed for at least 5 minutes for further removal of milk from the explants. This point in the experimental protocol was reached not more than one and a half hours after harvesting of the mammary tissue.

The mammary explants were then harvested from the buffer by filtration through a plastic sieve and washed with 4 ml ice cold buffer. Portions (up to 50 mg of explants per vial) of explants were then transferred to 20 ml scintillation vials containing 4 ml of the experimental buffer and incubated for a further 2 minutes at 37 °C prior to the addition of radiolabelled amino acids. Explants were gassed with O_2 at frequent intervals during the incubations.

3.2.5. Determination of extracellular space and tissue dry matter in mammary explants

The extracellular space in explants was determined in parallel experiments to the determination of amino acid uptake. It has previously been established that sucrose is a good extracellular marker in mammary tissue (Linzell and Peaker, 1971). Thus, [^{3}H]sucrose was used as the extracellular marker. The accumulation of radiolabel by mammary tissue explants was determined as for amino acid uptake (see section 3.2.6). The

sucrose space, and hence the extracellular space, was determined from the proportion of radioactivity per g of tissue compared with the radioactivity per ml of incubation medium according to equation 3.1:

$$F = \text{DPM sucrose}_t / \text{DPM sucrose}_m \quad (3.1)$$

where F is the proportion of the wet mass of mammary tissue that was accessible to radiolabelled sucrose, and hence the extracellular space, DPM (Disintegrations Per Minute) sucrose_t is the radioactivity associated with the mammary tissue in DPM / mg tissue and DPM sucrose_m is the radioactivity associated with the incubation medium in DPM / µl incubation medium.

3.2.6. Determination of amino acid uptake by mammary explants

Uptake of amino acid was determined using L-[^{3}H]-amino acids as tracers (0.25 - 0.5 µCi / ml). Mammary explants were incubated with one of these tracers for 2 minutes at 37 °C, after which time the tissue was removed from the incubation vial to a plastic sieve. The explants were then washed with 4 ml of ice cold buffer (same composition as in section 3.2.2), removed from the sieve and lightly blotted on filter paper (Whatman's No.1). The explants were transferred to pre-weighed vials that were re-weighed to determine the weight of tissue. Following this, 4 ml of a 10 % trichloroacetic acid (TCA) solution was added to each tube which was then left to stand for at least 16 hours at room temperature to allow the radio-isotope contained in the tissue to leach out. The tubes were then centrifuged at 13,000 g for 2 minutes and 1 ml of the resulting supernatant added to 10 ml of UltimaGold scintillation fluid. Radioactivity was counted using a scintillation counter (Tri-Carb 2250CA Liquid Scintillation Analyser; Canberra Packard, Pangbourne, Berks., UK). The specific activity (DPM / nmole) of the radioisotopes in the incubation medium was determined by counting the radioactivity in 100 µl aliquots of each incubation

medium. The specific activity was used to calculate the concentrations of amino acids within mammary tissue according to equation 3.2:

$$[\text{amino acid}]_c = ([\text{amino acid}]_t - [\text{amino acid}]_m \cdot F)/(1-F) \quad (3.2)$$

where $[\text{amino acid}]_c$ is the cellular concentration of amino acid in nmol / g cells, $[\text{amino acid}]_t$ is the total tissue concentration of amino acid in nmol / g of tissue wet weight, $[\text{amino acid}]_m$ is the concentration of amino acid in the incubation medium in nmol / ml, F is the sucrose space expressed as a proportion of the tissue wet weight determined over the same timed period as the corresponding uptake.

3.2.7. Experimental procedures

Lactating rat mammary tissue explants were prepared as described previously and incubated at 37 °C for 20 minutes in an oxygenated buffer (see section 3.2.3 for the composition) containing 5 μM of one of the amino acids, L-glutamate, L-histidine, L-leucine and L-lysine prior to the addition of the L-[^{3}H]-amino acids. Following this, at predetermined times, mammary tissue fragments were removed from the buffer, washed and the tissue weight was determined. Amino acid uptake was measured as described previously and expressed as nmoles / g of tissue.

For the preliminary experiments, the time course of L-leucine and the sodium dependence of L-glutamate and L-histidine uptake by lactating rat mammary tissue explants were measured to allow comparison with corresponding data in the literature. The effects of addition of L-leucine, BCH, L-glutamine, L-histidine and L-lysine, each at a concentration of 20 mM, on the uptake of L-histidine were also examined to determine the feasibility of the technique.

For the experiments designed to determine the effect of β-casomorphins, one of three β-casomorphins (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7) was added to the buffer at a concentration of 100 nM.

3.2.8. Statistical analysis

Statistical analysis was performed on the data using Genstat 5 (Payne *et al.*, 1993) and differences between treatments were determined by ANOVA and were considered significant when $P < 0.05$.

3.3. RESULTS

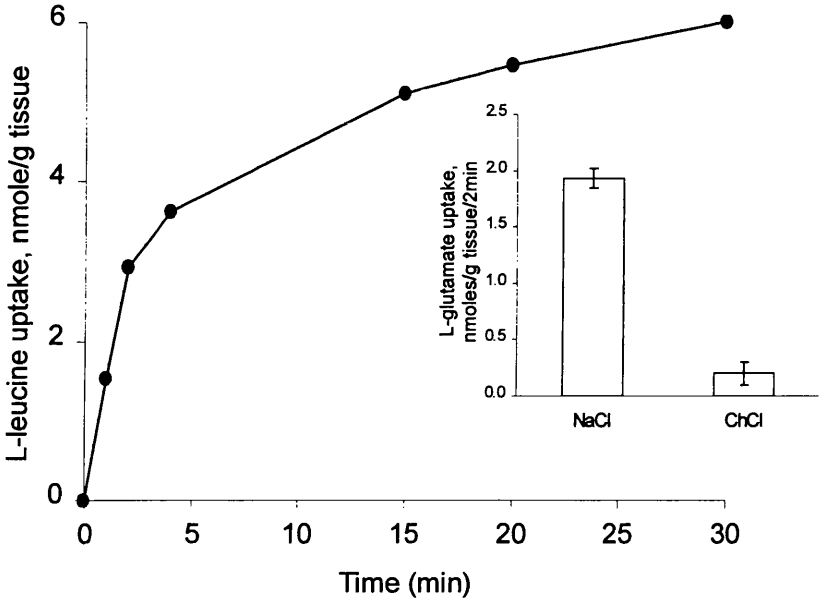
3.3.1. Characteristics of the amino acid uptake

Previous work has shown that L-glutamate and L-lysine uptake by rat mammary explants is linear for at least 2 minutes (Shennan *et al.*, 1994; Millar *et al.*, 1996). Therefore, in the present experiments the uptake of each amino acid after 2 minutes of incubation was used as an initial rate of influx. However, there are no data concerning the time-course of L-leucine and L-histidine uptake by rat mammary tissue and, in view of this, experiments were conducted to investigate the time dependence of L-leucine and L-histidine uptake by rat mammary tissue explants. In addition, the ion dependence of L-leucine and L-glutamate was studied.

Figure 3-1a shows the time-course of L-leucine uptake in the presence of extracellular Na^+. It is evident that L-leucine influx is time-dependent being linear for at least 2 minutes. Figure 3-1b shows that L-histidine uptake by rat mammary explants is also time-dependent but not a Na^+-dependent process. Thus, replacing extracellular Na^+ with choline had no significant effect. In contrast, replacing Na^+ with choline markedly reduced L-glutamate uptake (inset).

Figure 3-2 shows the effect of the amino acids (L-leucine, BCH, L-glutamine, L-histidine and L-lysine) at a concentration of 20 mM on the uptake of L-histidine. It is evident that the mammary tissue explants with 2-minute incubations can be a convenient and effective method for the measurement of amino acid uptake.

a)



b)

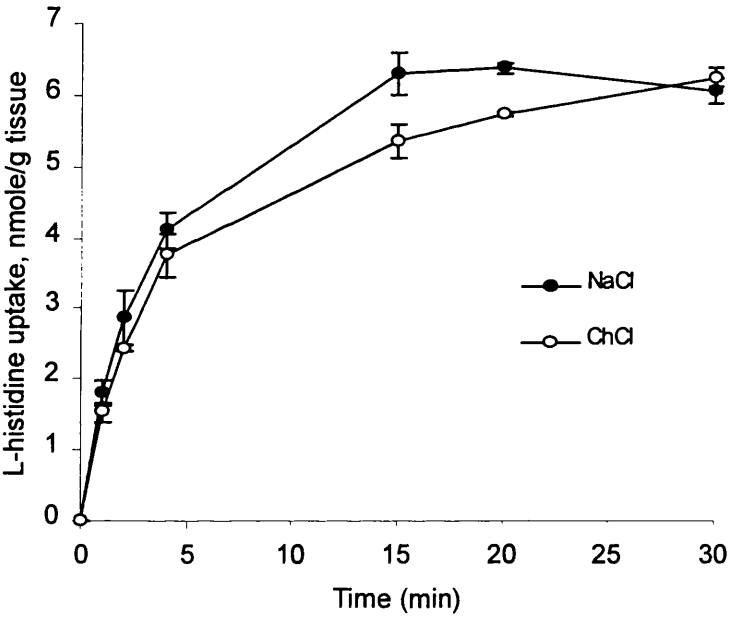


Figure 3-1. a) Time course of L-leucine and sodium dependence (inset) of L-glutamate uptake by lactating rat mammary tissue explants. Na^+ in the buffer was replaced with choline for the sodium dependence experiment. Values for the time course of L-leucine are from the tissue of a single animal and, for the inset, are from tissue of a single animal with the incubation performed in triplicate with SEM shown by a vertical bar. b) Time course of L-histidine uptake by lactating rat mammary tissue explants with and without NaCl in the buffer. All points are means for three experiments using tissue from separate animals with SEM shown by vertical bars.

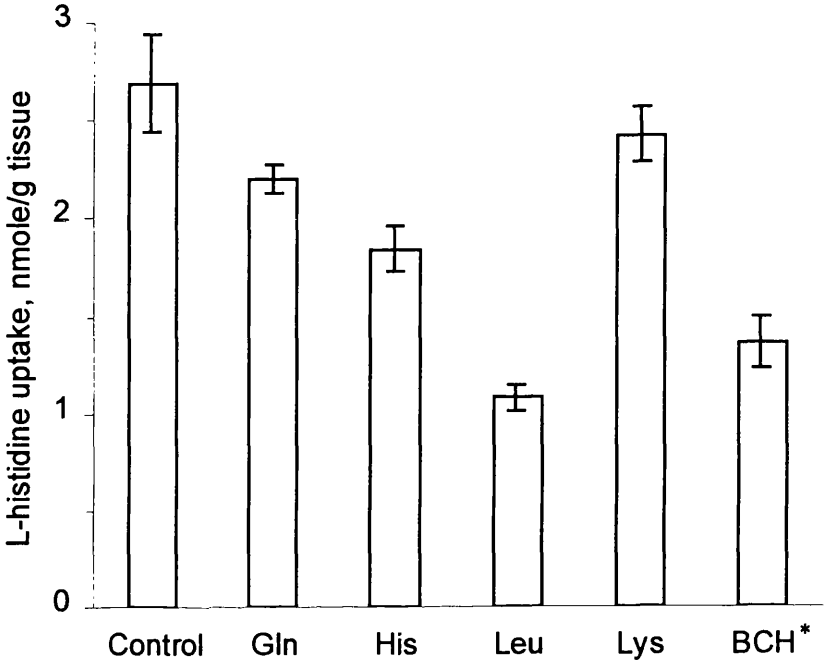


Figure 3-2. Effect of amino acids on L-histidine uptake by lactating rat mammary tissue explants in the presence of external Na^+. Values are means for four experiments using two animals with SEM shown by vertical bars. L-histidine uptake was assayed after 2-minutes incubation.

* 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid

3.3.2. Effect of β-casomorphins on the amino acid uptake

Table 3-1 shows the effect of β-casomorphins on L-glutamate, L-histidine, L-lysine and L-leucine uptake where data are expressed as nmole / g of tissue with SEM (Standard Error of the Means). The effects of β-casomorphins were tested on the initial rate of amino acid uptake. The results of the experiments are also summarised in figure 3-3. The addition of β-casomorphins to the incubation medium failed to affect the uptake of the amino acids by the lactating rat mammary tissue explants.

3.4. DISCUSSION

The accumulation of leucine in everted sacs of the rat jejunum was increased when β-casomorphin-5 or its synthetic analogue, [D-Ala^2] β-casomorphin-5-NH_2, was coincubated with the amino acid (Ermisch *et al.*, 1989). As an initial step in the overall study of the possible involvement of β-casomorphins in milk production responses to abomasal infusions of casein, *in vitro* experiments were carried out using lactating rat mammary tissue explants to test the effects of β-casomorphins on amino acid uptake by the mammary gland.

Mammary tissue explants provide a convenient and viable model for studying amino acid transport, particularly the basolateral aspect of the transport, by lactating mammary cells (see Shennan *et al*, 1997). In the experiments designed to detect the ability of β-casomorphins to alter the mammary uptake of representative amino acids (two neutral, one anionic and one cationic amino acid), there was no evidence of action of the peptides on amino acid uptake by mammary tissue. It might be argued that the technique applied to the study was not sensitive enough to detect a possibly small effect of the β-casomorphins. However, examination of the results of Ermisch *et al.* (1989) shows that the effects on amino acid uptake were evident by 1 minute (i.e. stimulation of 80 to 100 %). It is, then, a

Table 3-1. Effect of β-casomorphin-4-amide (CM-4-NH_2), β-casomorphin-5 (CM-5) and β-casomorphin-7 (CM-7) on the uptake (nmole / g tissue) of L-glutamate, L-histidine, L-leucine and L-lysine by lactating rat mammary tissue explants in the presence of external Na^+. Values are means ± SEM for three measurements using separate animals. Each experiment was carried out in triplicate. All the amino acid uptakes were assayed after 2-minute incubation.

	Experiment 1			Experiment 2	
	Control	CM-4-NH_2	CM-7	Control	CM-5
Glutamate	2.11 ± 0.42	1.85 ± 0.52	1.94 ± 0.50	2.95 ± 0.07	2.71 ± 0.13
Histidine	3.00 ± 0.30	2.86 ± 0.45	2.92 ± 0.38	2.29 ± 0.16	2.37 ± 0.35
Leucine	3.19 ± 0.24	3.22 ± 0.33	3.43 ± 0.44	2.31 ± 0.05	2.16 ± 0.14
Lysine	2.60 ± 0.13	2.60 ± 0.04	2.66 ± 0.04	2.48 ± 0.37	2.76 ± 0.38

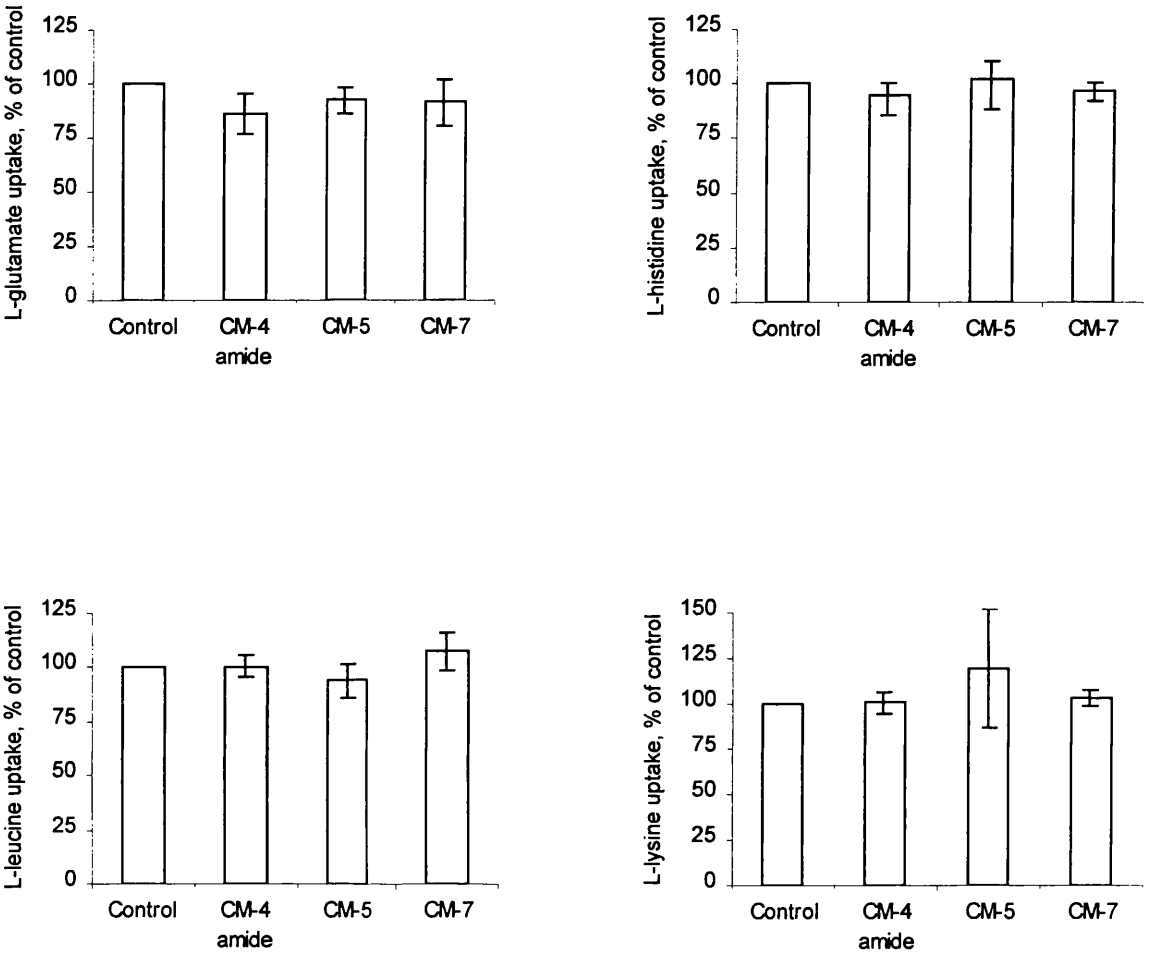


Figure 3-3. Effects of β-casomorphin-4-amide (CM-4 amide), β-casomorphin-5 (CM-5) and β-casomorphin-7 (CM-7) on the uptake of L-glutamate, L-histidine, L-leucine and L-lysine by lactating rat mammary tissue explants in the presence of external Na^+. Values for each amino acid uptake are expressed as percentage of controls and means for three experiments using separate animals with SEM shown by vertical bars. All the amino acid uptakes were assayed after 2-minutes incubation.

fair assumption that the technique used in this study, which is an equivalent *in vitro* preparation for the mammary gland, should be able to detect any similar action of the peptides on amino acid uptake.

It was suggested (Ermisch *et al.*, 1989) that the stimulation of L-leucine uptake in the small intestine by β-casomorphins is via their interaction with opioid receptors at the luminal membrane of the enterocytes. The lack of response in the present study may be due to the absence or low number of the opioid receptors in the mammary tissue. Although opioid receptors were expressed in the human mammary cancer cell line A431 (Ammer and Schulz, 1997), there is no direct evidence of the existence of opioid receptors at the surface of mammary epithelial cells whereas the small intestine is regarded as one of the major sites of the receptors (Dashwood *et al.*, 1985; Nishimura *et al.*, 1986). Furthermore, even if opioid receptors do occur in mammary tissue, the effects on amino acid uptake presumably require a specific receptor type, which may not be among those present.

Different cell types have different distributions of amino acid transport systems to meet the specific demands of each cell type. For example, the transport of L-leucine in the brush-border membrane vesicles prepared from guinea-pig small intestine is largely Na-dependent with a small fraction of Na^+-independent transport (Satoh *et al.*, 1989) whereas, in the mammary gland, its uptake is largely Na^+-independent (Figure 3-4 or ref). Furthermore, it has been suggested that there may be mammary tissue variants of the system L, the major Na^+-independent system for L-leucine (see Shennan *et al.*, 1997). Therefore, the differences in the conformation of transport systems in the mammary tissue may also be, at least in part, responsible for the result.

Overall, it is concluded that direct action of β-casomorphins on mammary amino acid transport is unlikely, although further investigations would be necessary before completely ruling out the possibility.

CHAPTER FOUR

THE SYNTHESIS AND ANALYSIS OF β-CASOMORPHINS AND THEIR RUMINAL DEGRADATION IN 'PROTECTED' AND ' UNPROTECTED' FORMS

4.1. INTRODUCTION

The results in Chapter 3 produced no evidence of actions of β-casomorphins on the mammary gland. Therefore the remaining experiments in this thesis were directed towards the effects of β-casomorphins at the level of the gut.

There were two major concerns in investigating the action of β-casomorphins in the ruminant. First, experiments in the ruminant, especially in the dairy cow, require large quantities of the peptides. Peptides with high purity can be obtained by solid phase peptide synthesis, which is easily carried out on a peptide synthesiser combined with FMOC (9-fluorenylmethoxycarbonyl) chemistry (Fields and Noble, 1990). However, synthesising large quantities of peptides with relatively high purity could become a time-consuming process and would require access to a peptide synthesiser with a single large or multiple smaller reaction vessels. Second, although the most convenient way of administering the β-casomorphins is by addition to the diet, the ability of the peptides to survive degradation in the rumen, and to reach the small intestine, was not known. Hence it was essential to study the fates of β-casomorphins in the rumen if dietary supplements of the peptides were to be used. β-casomorphins, like proline-rich peptides in general, display remarkable resistance to the attack of pancreatic proteases of simple-stomached animals *in vitro* (Brantl and Teschemacher, 1979). However, there was no information available on their stability against hydrolysis by enzymes of microbes in the rumen.

In investigating the fate of β-casomorphins in the rumen, another concern was that their quantitative as well as qualitative analysis in mixed rumen samples might be

problematic, not only because of their high hydrophobicity but also because of the presence of background concentrations of other nitrogenous compounds (e.g. small peptides) in the rumen samples, which would be expected to interfere with the analysis.

The aims of the present study were to synthesise β-casomorphins in gram quantities; to develop methods of detecting β-casomorphins in mixed rumen samples; and to determine their stability in the rumen. As the first experiment revealed that the β-casomorphins are readily degraded in the rumen *in vitro*, an attempt was also made to chemically protect the peptides from ruminal hydrolysis.

4.2. MATERIALS AND METHODS

4.2.1. HPLC (High-performance liquid chromatography) analysis

The concentration of the individual peptides was determined by HPLC using a reverse-phase C18 column by modifications of the methods of Wallace & McKain (1989) and Muehlenkamp *et al.* (1996). The HPLC apparatus used was a LDC / Milton Roy system (Riviera Beach, Florida, USA) fitted with Spherisorb S5 ODS (5 μm, C18; LDC / Milton Roy) column (250 by 4.6 mm) with a 20 μl loop. The peptides were obtained from Calbiochem-Novabiochem (UK) Ltd. All chemicals and reagents were HPLC grade and were supplied by Sigma Ltd.

Effects of organic solvent on retention time of peptides

To determine the effects of different ratios of organic solvent in the eluant on the retention time of peptides, three peptides (Ala-Ala, Ala-Ala-Ala and Ala-Ala-Ala-Ala) were eluted in a mixture of solvent A (30 mM orthophosphoric acid) and solvent B (methanol) in the ratios of 0:100, 10:90, 20:80 and 30:70 (solvent A:solvent B).

Effects of ion-pairing reagent on retention time of peptides

To determine the effects of ion-pairing reagents in the eluant on the retention time of peptides, three peptides (Ala-Ala, Ala-Ala-Ala and Ala-Ala-Ala-Ala) were eluted in a mixture of 60 %, 30 mM orthophosphoric acid and 40 % methanol with heptane sulfonic acid (HSA) as the ion-pairing reagent at the concentrations of 0, 5, 10 and 50 mM.

HPLC of β-casomorphins

For the analysis of the β-casomorphins in mixed rumen samples, optimal flow rate and eluant compositions, including concentrations of the ion-pairing reagent were determined from elution profiles for the peptides and rumen samples determined under a range of conditions. The flow rate was 1.2 ml / min for β-casomorphin-4-amide and β-casomorphin-5 and 1.0 ml / min for β-casomorphin-7, and the detector was set at 206 nm for β-casomorphin-4-amide and β-casomorphin-5 and 220 nm for β-casomorphin-7. The eluant for the analysis of β-casomorphin-4-amide was a mixture of 60 %, 30 mM orthophosphoric acid plus 2 mM HSA as the ion-pairing agent and 40 % methanol. For β-casomorphin-5, the eluant was as for β-casomorphin-4-amide except that 5 mM HSA concentration was used. β-casomorphin-7 was eluted in a mixture of 70 % solvent A (0.1 % triflouroacetic acid, TFA and 99 % HPLC grade water) and 30 % solvent B (0.1 % TFA, 90 % acetonitrile and 9.9 % HPLC grade water).

4.2.2. Chemical synthesis of β-casomorphins

The peptides (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7) used in the experiments were synthesised by solid-phase peptide synthesis using FMOC chemistry (see Fields and Noble, 1990 for detailed information). The peptide synthesiser used was BT 7500 Solid Phase Peptide Synthesiser (Biotech Instrument Ltd, Luton, UK) with a 45 ml reaction vessel.

Figure 4-1 gives a brief outline of the process. At each coupling step, a small amount of resin from the reaction vessel was taken and subjected to the Kaiser test (see below) in order to confirm the successful coupling of each sequence. After the coupling process was completed, peptides were cleaved and all side-chain protection groups were deprotected from the resin by incubating in a cleavage reagent at room temperature for 2 hours and filtering through a sintered glass funnel under positive pressure of N_2. The composition of the cleavage reagent was 90 % (v/v) TFA, 2.5 % (v/v) H_2O, 2.5 % (v/v) thioanisole, 1.25 % (v/v) 1,2-ethanedithiol and 3.75 % (w/v) phenol. The filtered cleavage solution was then transferred to a round-bottomed flask and was evaporated using a vacuum evaporator at 60 °C for 30 minutes. Peptides were extracted by the cold ether extraction method. Briefly, 20 ml of ice cold ether was slowly added to the evaporated filtrate in a drop-wise manner. After the peptides were successfully extracted, all contents were transferred to a 20 ml centrifuge tube with a cap and centrifuged at 1500 g for 5 minutes at 4 °C. The pellet was resuspended very gently (finger vortexing) in 10 ml cold ether and centrifuged at 1500 g for 5 minutes at 4 °C. The process was repeated 5 times before drying the pellet in a dessicator overnight.

All resins, FMOC amino acids and TBTU (2-[1H-Benzotriazol-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate) were obtained from Calbiochem-Novabiochem (UK) Ltd. (Beeston, Nottingham, UK) and all other chemicals and reagents were obtained from Sigma Ltd.

Kaiser test

A small amount (about 10 mg) of samples from the reaction vessel of the synthesiser was transferred into a glass tube containing 2 ml of 0.1 mM potassium cyanide. The tube was heated in a block thermostat at 100 °C for 5 minutes. The reactivity of samples, which was proportional to colour intensity, was accessed by eye.

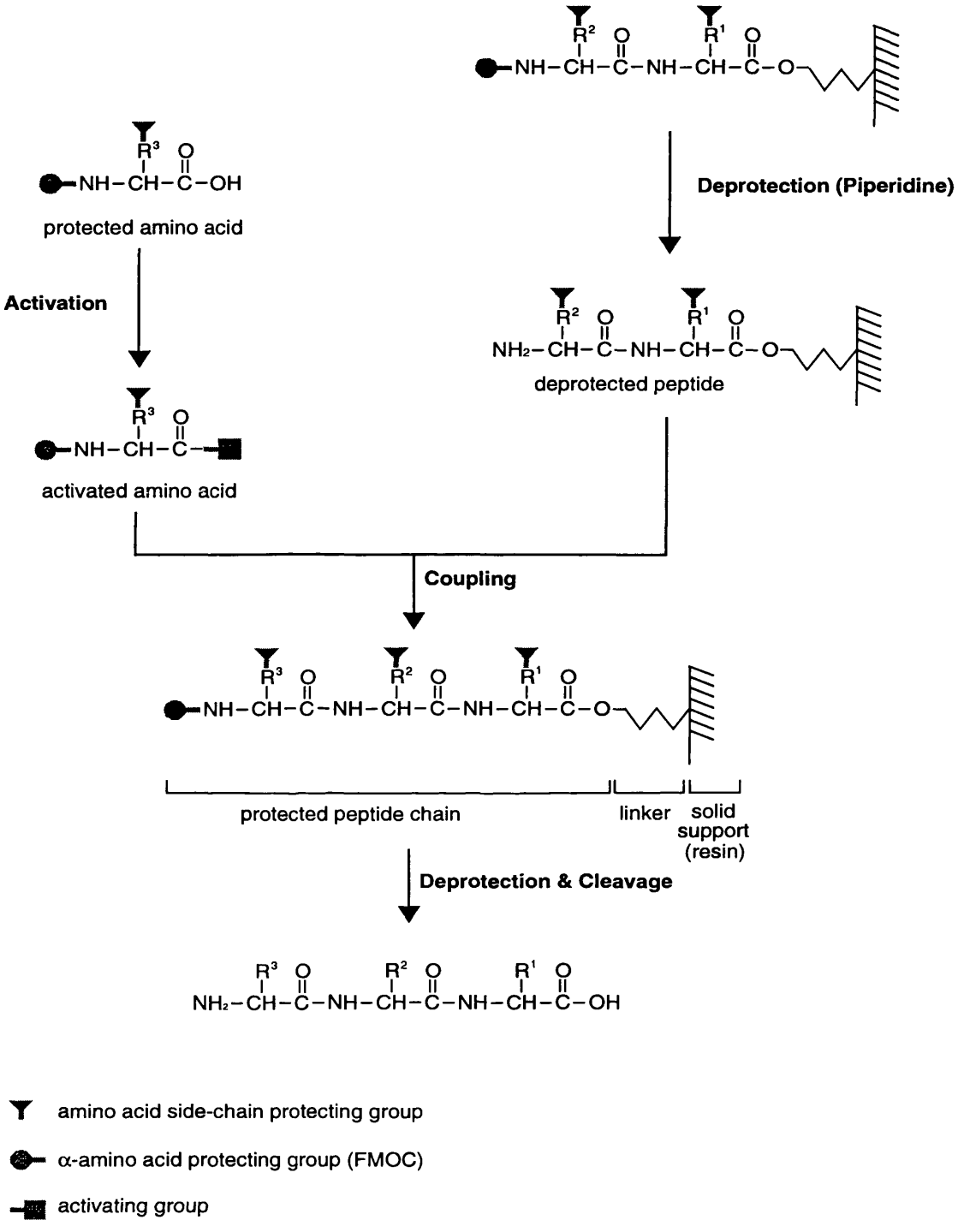


Figure 4-1. Solid Phase Peptide Synthesis

4.2.3. MALDI-TOF-MS (Matrix-assisted laser desoption/ionisation time-of-flight mass spectrometer)

To authenticate synthesised peptides, 1 mg of samples from extracted crude peptides was dissolved in 1 ml of matrix solution and their molecular weight was determined by MALDI-TOF-MS (figure 4-2). The matrix used was α-Cyano hydroxycinnamic acid and the matrix solution was 0.1 % TFA in 70 : 30 acetonitrile : water. 1 μl aliquots of samples and matrix were pipetted onto a metal target slide and allowed to air-dry (approximately 5 minutes). Peptide spectra were obtained using a Finnigan MAT (Hemel Hempstead, Hertshire, UK) LaserMat 2000 time-of-flight mass spectrometer.

4.2.4. Acetylation of β-casomorphins

N-terminal acetylation of the peptides was carried out using acetic anhydride according to the method of Means and Feeney (1964). 5 mg of each of the peptides was dissolved in distilled water at a concentration of 2 % (w/v) and chilled in an ice-water slurry. Acetic anhydride was added to the peptide solution to a concentration of 1 M and the mixture was incubated on ice for 1 hour. Solutions of acetylated peptides were dried using a centrifugal evaporator, then resuspended in distilled water (300 μl) and freeze-dried.

To measure the success rate of the acetylation, α-NH_2-N measurement of the acetylated peptides and the non-acetylated peptides was performed using a ninhydrin method (Moore and Stein, 1954).

4.2.5. Protein assay of rumen fluid samples

Protein concentrations of the rumen fluid samples from each sheep were determined by a modified Lowry method as follows.

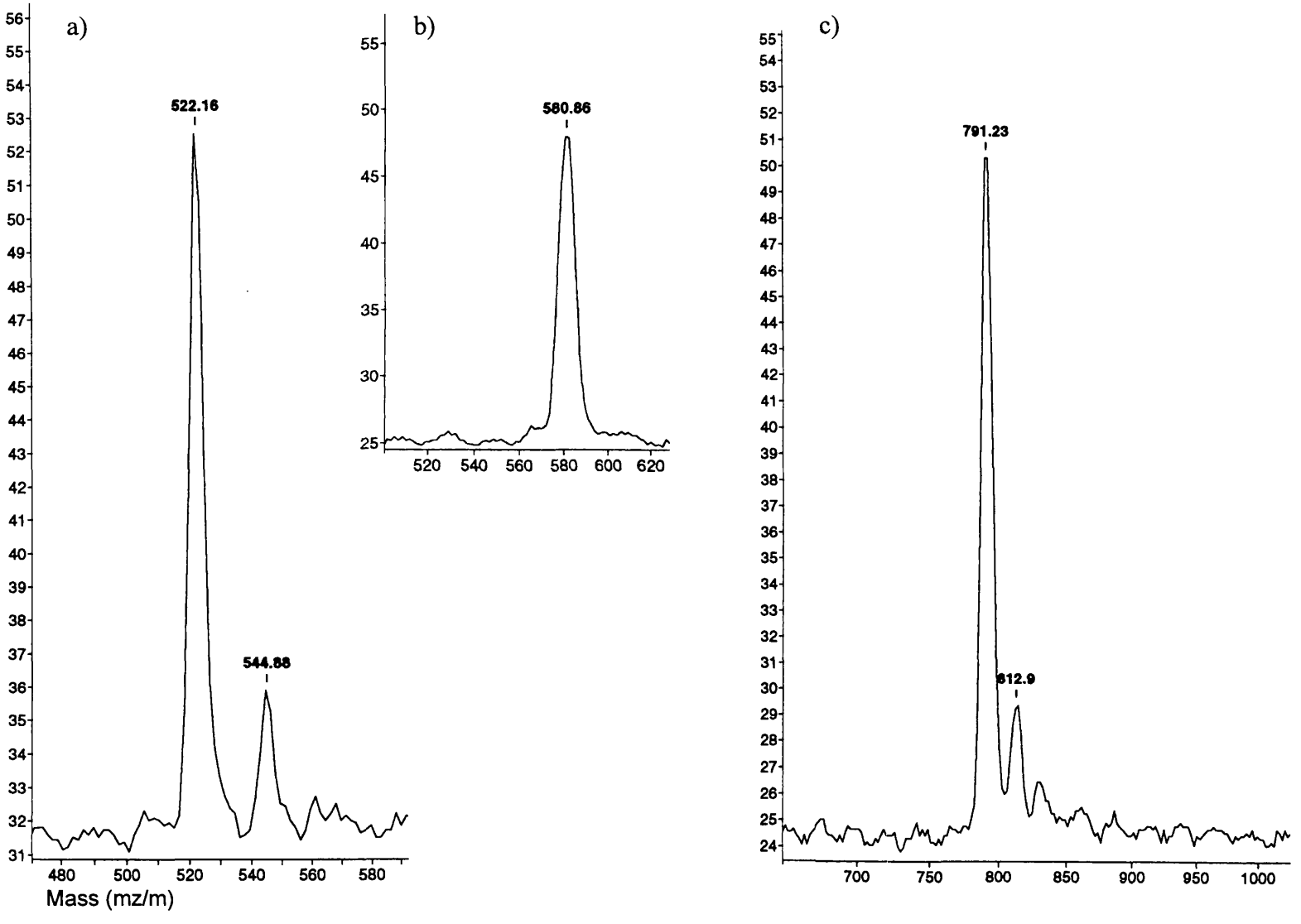


Figure 4-2. MALDI analysis of a) β-casomorphin-4-amide, b) β-casomorphin-5 and c) β-casomorphin-7

Reagents

Lowry B solution: 1 % potassium sodium tartrate + 0.5 % $CuSO_4 \cdot 5H_2O$, pH 7.0

Reagent 1: 50 ml 5 % Na_2CO_3 + 2 ml Lowry B solution

Reagent 2: Folin Ciocalteu reagent diluted 1:1 in distilled water

Procedure

1 ml of rumen fluid samples was added to a 1.5 ml eppendorf tube containing 0.25 ml 25 % (v/v) TCA which was then centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded and the remaining pellet was resuspended in 1 ml 0.5 M NaOH. Standards (0, 0.04, 0.08, 0.12, 0.16 and 0.2 mg / ml) which were prepared using BSA and diluted samples (1:50) were boiled for 5 minutes in a boiling water bath and cooled at room temperature. 50 µl of samples and standards were transferred to a microtitre plate and 1.25 µl of reagent 1 was added to each well which was then incubated at room temperature for 10 minutes before 25 µl of reagent 2 was added to each well. The plate was incubated at room temperature for 30 minutes and OD values were read at 700 nm on a MR 5000 plate reader (Dynex Laboratories, Billinghurst, West Sussex, UK).

4.2.6. Experiment 1. Rumen incubation of β-casomorphins

Animals

Four adult sheep, fitted with permanent rumen cannulas, received a maintenance diet of hay, barley, molasses, fish meal and vitamins-minerals mixture (500, 299.5, 100, 91 and 9.5 g / kg dry matter respectively). Samples of rumen fluid were removed 3 hours after feeding. These samples were strained through four layers of muslin and used immediately.

Rumen incubation and sampling

β-Casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7 were added to strained rumen fluid from each of the four sheep to a final peptide concentration of 0.125 mM in a total incubation volume of 10 ml, and the mixtures were incubated under carbon dioxide in stoppered containers in a shaking water-bath at 39 °C. Samples (1.0 ml) were removed after 0, 5, 10, 30, 60 minute(s), 2 and 6 hours into microcentrifuge tubes containing 0.25 ml phosphoric acid (1.25 M). Tubes were chilled at 4 °C and centrifuged at 12,000 g for 5 min at 20 °C and the supernatant fluid was stored at -20 °C until analysis. Peptide analysis was done on the supernatant fluid filtered through 0.45-μm-pore-size membrane filters. Owing to the accidental breakage of one container before the incubation, data from one sheep were excluded and data are expressed as means of values from three sheep.

4.2.7. Experiment 2. Rumen incubation of N-terminal acetylated and non-acetylated β-casomorphins

Animals

Four adult sheep, fitted with permanent rumen cannulas, received the same diet as described in the Experiment 1. Samples of rumen fluid were removed 5 hours after feeding. These samples were strained through four layers of muslin and used immediately.

Rumen incubation and sampling

Rumen incubations of three β-casomorphins (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7) and their corresponding acetylated peptides were carried out by the procedure described for the Experiment 1 except that samples were removed after 0, 15, 30, 60, 90 minute(s) and 6 hours.

4.3. RESULTS

4.3.1. Solid phase peptide synthesis

0.6 mmole of the β-casomorphins (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7) were produced by one synthesis cycle. The average recovery rate of the peptides after the extraction was 80 %.

4.3.2. HPLC of peptides

Effects of methanol and HSA concentrations on the retention time of peptides.

The retention times of alanine oligopeptides with different concentrations of methanol and HSA in the mobile phase are shown in table 4-1 and table 4-2 respectively. The higher methanol content of the eluant resulted in a shorter retention time of the alanine oligopeptides. Increasing the concentration of HSA in the mobile phase delayed the retention times of the peptides.

It was found that, by altering combination of the two components of the mobile phase, the selectivity of the reversed-phase HPLC system for oligopeptides could be increased.

HPLC analysis of β-casomorphins in rumen fluid

The elution profiles of β-casomorphins and a typical rumen fluid sample from two different eluants are shown in figure 4-3. There was no interference between β-casomorphin-7 and other compounds found in rumen fluid in either eluant conditions whereas β-casomorphin-4-amide and β-casomorphin-5 appear to be subject to interference from other compounds in the rumen fluid in both eluant conditions.

Table 4-1. Effects of different concentrations of methanol on the retention time (min) of Ala_n. Values are means of two observations.

Peptide	Methanol Concentration (%)			
	0	10	20	30
Ala_2	11.4	5.9	3.8	2.9
Ala_3	22.7	8.1	4.4	3.0
Ala_4	34.3	10.0	4.9	3.1

Table 4-2. Effects of adding different concentrations of the ion-pairing reagent (HSA, heptane sulfonic acid) on the retention time (min) of Ala_n. Values are means of two observations.

Peptide	HSA Concentration (mM)			
	0	5	10	50
Ala_2	1.92	3.82	4.15	5.20
Ala_3	2.12	4.39	4.62	5.60
Ala_4	2.22	4.92	4.99	5.72

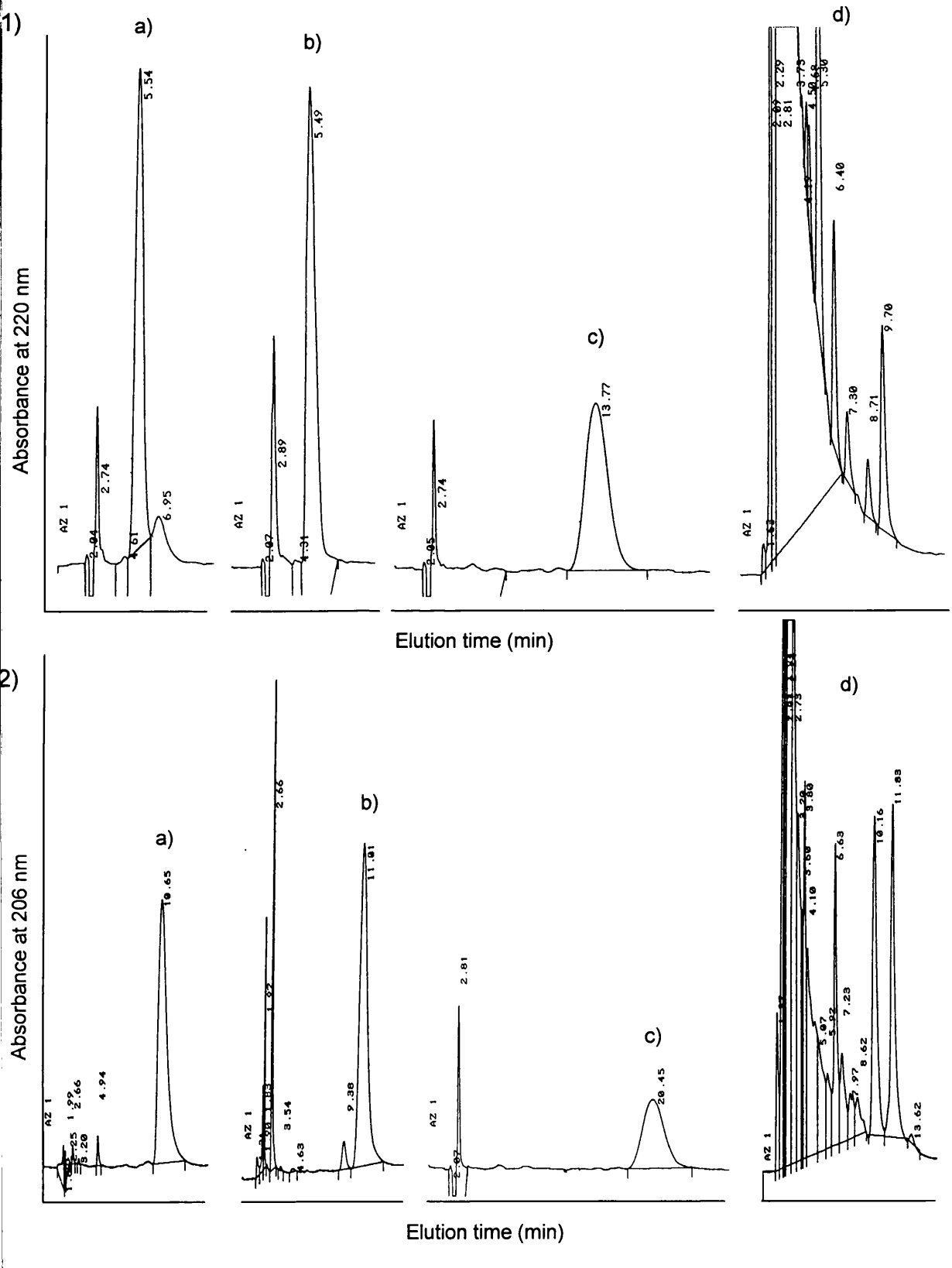


Figure 4-3. Elution profiles of a) β-casomorphin-4-amide, b) β-casomorphin-5, c) β-casomorphin-7 and d) a typical rumen liquid sample. Flow rate: 1) 1.0 ml / min, 2) 1.2 ml / min. Mobile phase: 1) 70 %, 0.1 % TFA in H_2O and 30 %, 0.1 % TFA and 9.9 % H_2O in acetonitrile and 2) 60 %, 30mM orthophosphoric acid and 40 % methanol.

The chromatograms shown in figure 4-4 are the profiles of β-casomorphin-4-amide, β-casomorphin-5 and rumen fluid samples containing the peptides where the compositions of the eluants were optimised to separate the peaks of the peptides from other peaks in the rumen fluid. It was possible to selectively alter the retention times of β-casomorphin-4-amide and β-casomorphin-5 with the inclusion of HSA at the concentrations of 2 mM and 5 mM respectively in order to avoid interference from other compounds in the rumen fluid, so enabling quantitative analysis of the peptides in mixed rumen samples.

4.3.3. Disappearance of β-casomorphins in rumen fluid

The rates of hydrolysis of three β-casomorphins (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7) were determined by incubating the peptides in rumen fluid *in vitro* and measuring the disappearance of the peptides after analysis by HPLC. All three peptides were rapidly broken down in the rumen fluid with half-lives of 20.7, 19.5 and 17.0 minutes respectively (figure 4-5).

4.3.4. Disappearance of N-terminal acetylated and non-acetylated β-casomorphins in rumen fluid

Average protein concentration of the rumen fluid samples was 2.36 mg / ml (SED = 0.384) which was very similar to that in the experiments of Wallace and McKain (1989, 2.31 mg / ml). Treatment of β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7 with acetic anhydride resulted in the blocking of 82.2, 92.6 and 93.5 % of N-terminus amino groups respectively, as determined by the ninhydrin method.

Figure 4-6 shows the pattern of hydrolysis of three β-casomorphins (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7) and their acetylated forms in rumen fluid. The rates of hydrolysis of the non-acetylated β-casomorphins were similar to those of Experiment 1 with half-lives of 17.6, 24.1 and 15.8 minutes respectively.

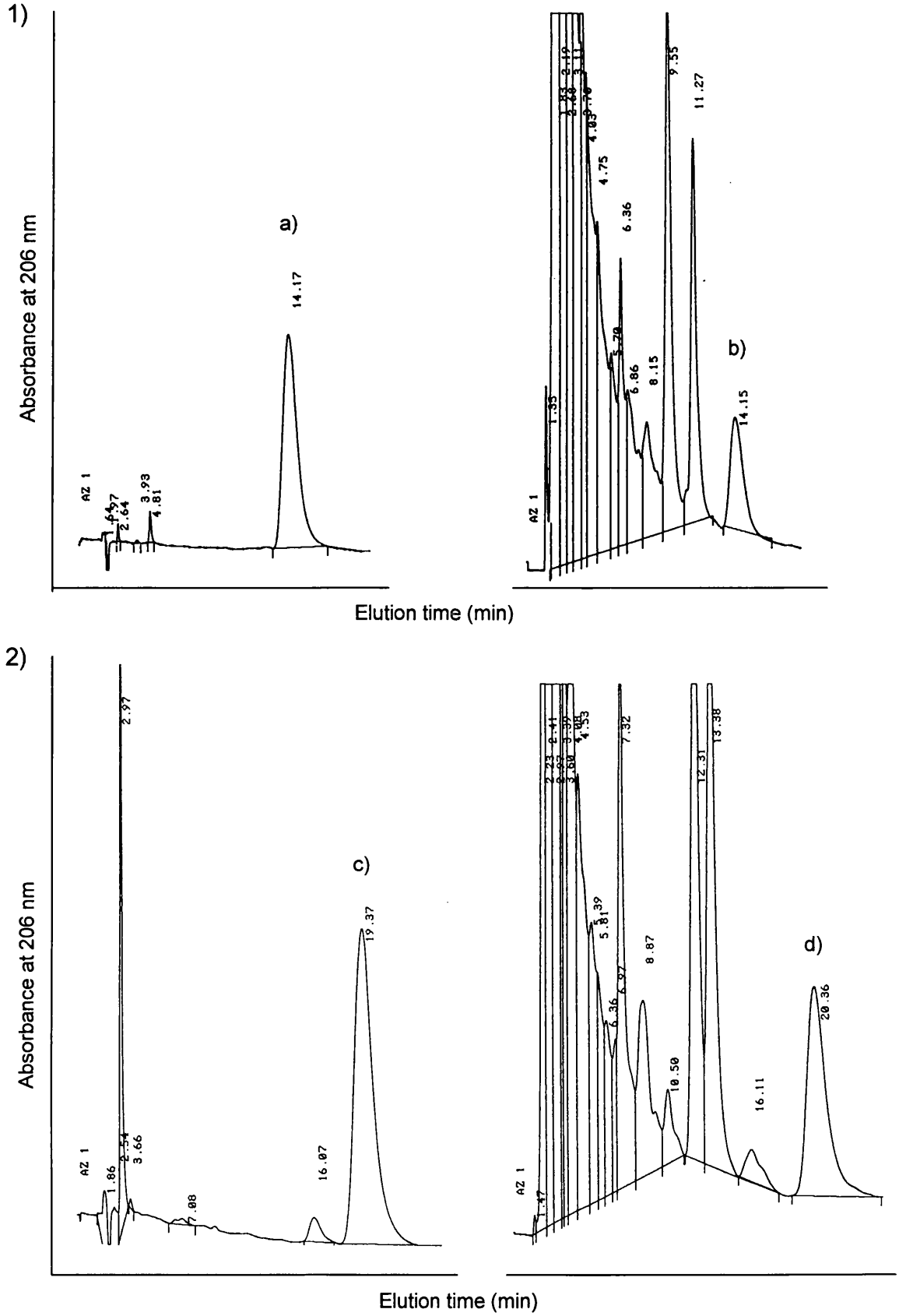


Figure 4-4. Elution profiles of a) β-casomorphin-4-amide (125 nmole / ml), b) β-casomorphin-4-amide(100 nmole / ml) in rumen liquid, c) β-casomorphin-5 (125 nmole / ml) and d) β-casomorphin-5 (100 nmole / ml) in rumen liquid. Mobile phase: 1) 60 %, 30mM orthophosphoric acid plus 2 mM HSA and 40 % methanol, 2) 60 %, 30mM orthophosphoric acid plus 5 mM HSA and 40 % methanol.

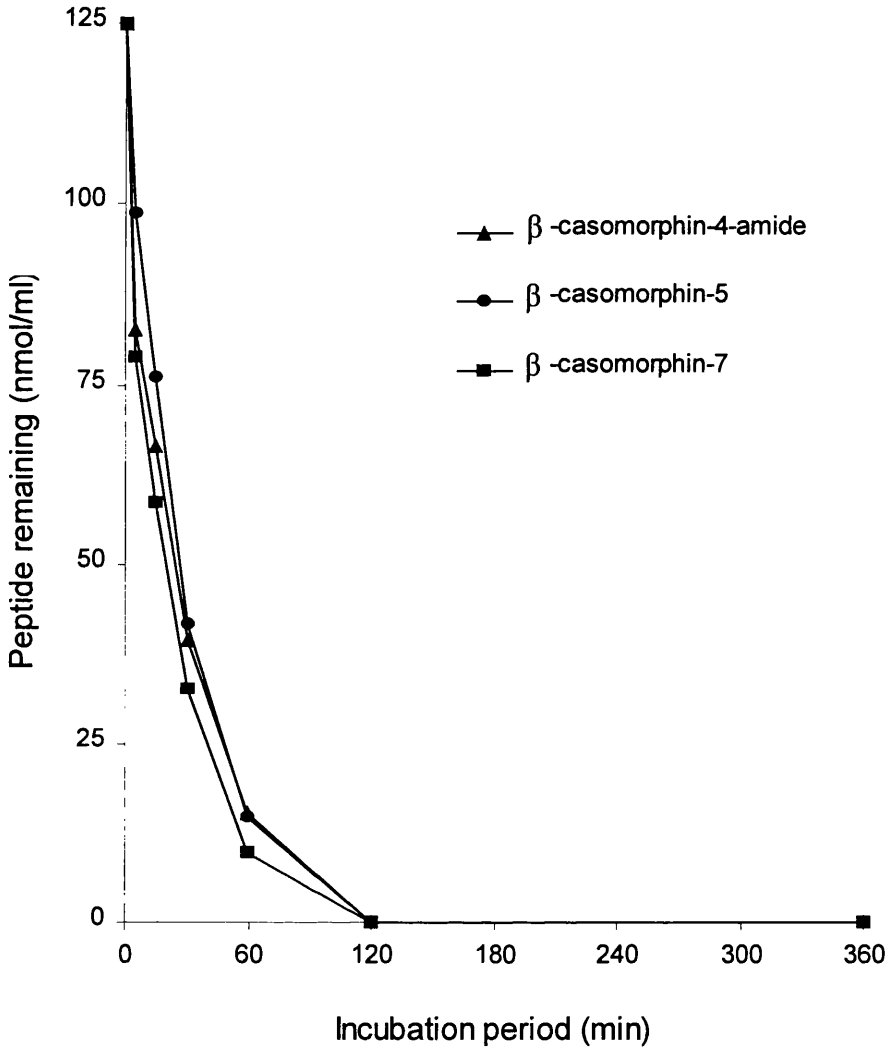


Figure 4-5. Disappearance of β-casomorphins added to rumen fluid *in vitro*. Results are means for samples of rumen fluid taken from three sheep.

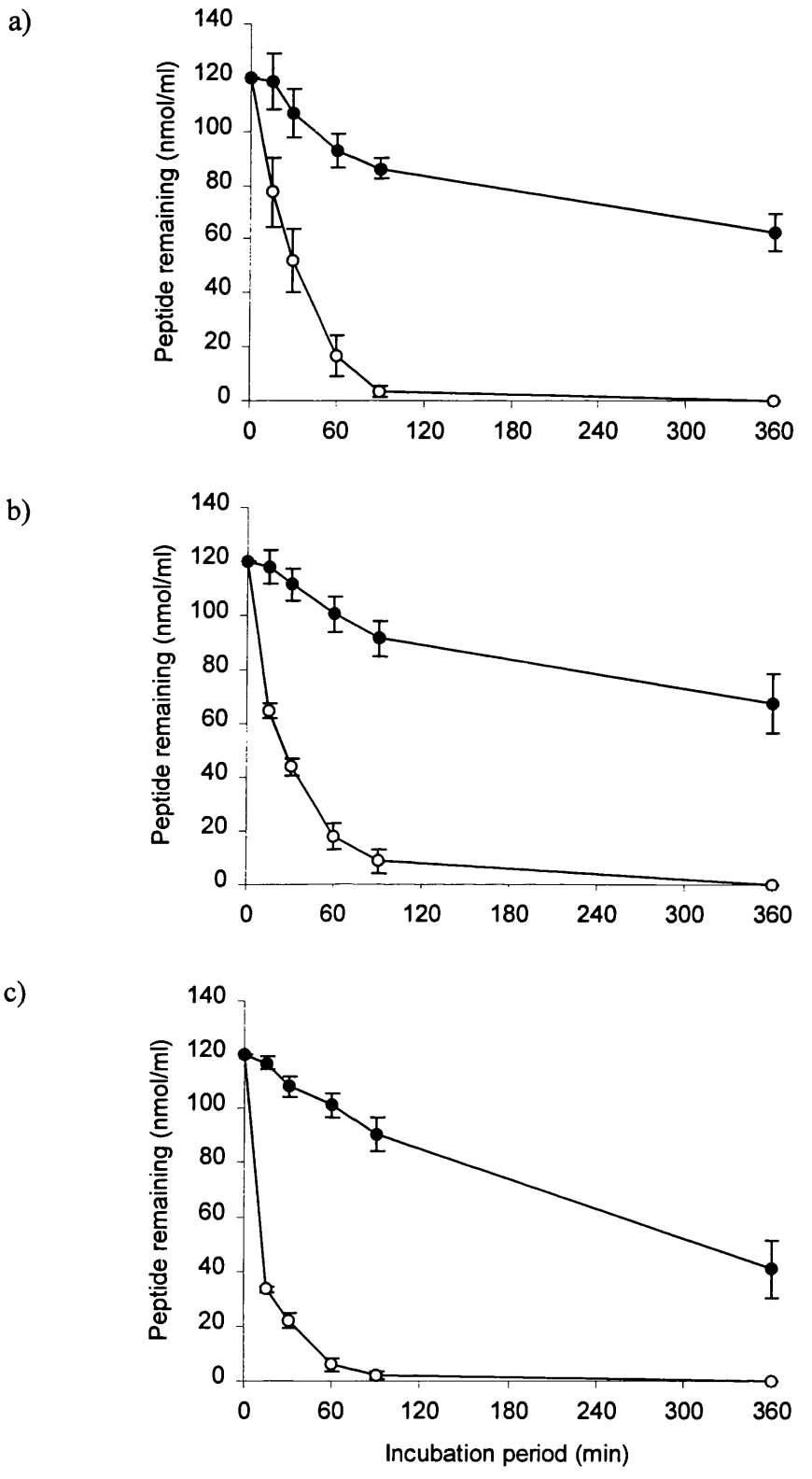


Figure 4-6. Disappearance of β-casomorphins (○) and N-acetyl β-casomorphins (●) added to rumen fluid *in vitro*. Results are means and SED represented by vertical bars for samples of rumen fluid taken from four sheep. a) β-casomorphin-4-amide, b) β-casomorphin-5 and c) β-casomorphin-7.

Acetylation of the β-casomorphins caused marked resistance to hydrolysis in rumen fluid with half-lives increased to 6.5, 7.5 and 4 hours respectively.

4.4. DISCUSSION

Since its development (Merrifield, 1963), solid phase peptide synthesis has become an essential tool in all areas of peptide research. An essential part of the investigation of the actions of β-casomorphins in the dairy cow was the synthesis of the peptides in large quantities with relatively high purity. This was achieved in the present study by solid phase peptide synthesis combined with more recent FMOC chemistry with a large reaction vessel coupled with the cold ether extraction of the peptides.

Investigations of actions of β-casomorphins in the ruminant at the level of the gut could be carried out by simply supplementing the peptides as dietary supplements if the peptides were relatively stable in the rumen, so allowing them to reach the site of action (i.e. small intestine of the ruminant). Proline-containing peptides (Yang and Russell, 1992) and peptides with glycine or proline as the N-terminal or penultimate N-terminal residue (Wallace *et al.*, 1990) were relatively more resistant to ruminal degradation *in vitro*. This is supported by an observation *in vivo* that peptides remaining in the rumen several hours after feeding tended to be enriched with glycine and proline (Wallace *et al.*, 1993c). Indeed, the rate of breakdown of a tetra-peptide (Gly-Pro-Gly-Gly) was 0.07 μmol / ml rumen fluid / h whereas another tetra-peptide (Ala-Ala-Ala-Ala) was hydrolysed at a rate of 1.18 μmol / ml rumen fluid / h (Wallace *et al.*, 1990). Therefore, it was expected that β-casomorphins, as proline-rich peptides with a penultimate N-terminal proline residue, would be stable in the rumen. However, the results of Experiment 1 show all three of the most potent β-casomorphins to be readily degraded in the rumen with half-lives of only 17 to 20 minutes. Such short half lives rule out a simple dietary addition of β-casomorphins as

an effective route of administration in the dairy cow unless the peptides can be protected from hydrolysis.

Peptide breakdown in the rumen depends on various factors including the amino acid sequence, molecular weight and solubility of the peptide and the composition of the microbial population. There seems to be a positive correlation between molecular size and hydrolysis rate (Copper and Ling, 1985). The more rapid breakdown of β-casomorphin-7 compared with the other two, shorter peptides in the present study (see table 4-3) may be because of its longer peptide chain, which would be expected to make it more susceptible to endopeptidase activities of the ruminal microbes. This difference becomes clearer when the breakdown rates of the N-terminal acetylated β-casomorphins are compared (table 4-3).

The mechanism of the breakdown of the β-casomorphins by rumen microorganisms is not known. However, studies indicated that the predominant mechanism for hydrolysis of peptides by ruminal microbes is a dipeptidyl aminopeptidase like activity which cleaved dipeptides from the N-terminus of the peptide chain (Wallace and McKain, 1989; Wallace *et al.*, 1990). Furthermore, *Prevotella ruminicola* was reported to be the only species among the common ruminal microbial species responsible for the activity (Wallace, 1996). Thus, considering that the experimental procedure and the composition of the diet used in the present study were the same as, or very similar to, those of the studies mentioned above, the rapid breakdown of the β-casomorphins is probably largely due to the dipeptidyl aminopeptidase activity of *P. ruminicola* in the rumen fluid, although the intermediate products of the breakdown were not determined.

In Experiment 2 of the present study, an attempt was made to protect the β-casomorphins from ruminal hydrolysis by chemical treatment of the peptides with acetic anhydride. The results indicate that blocking the N-terminus of β-casomorphins could be a simple and effective method to protect the peptides from ruminal hydrolysis leading to 52.2, 56.3 and 34.1 % respectively remaining after 6 hours. The effectiveness of N-

terminal acetylation of the peptides is consistent with the earlier hypothesis that the dipeptidyl aminopeptidase activity of *P. ruminicola* is the predominant mechanism for the hydrolysis of the peptides.

Protection of nutrients from ruminal hydrolysis or fermentation is not a new concept. Proteins and limiting amino acids have been subjects of rumen protection for decades (Chalupa, 1975, 1980; Mangan *et al.* 1980; Casper *et al.*, 1987). Heat and chemical treatments were the major approaches to achieve rumen bypass in early studies because these methods are relatively simple and cost-effective (Vicini *et al.*, 1983; Lynch *et al.*, 1987). Other methods include the use of low solubility peptides or amino acid analogues (Papas *et al.*, 1974) and the use of lipids as a protective matrix for proteins (Palmquist, 1984). Protection of peptides received little attention until information on mechanisms of peptide metabolism accumulated (Copper and Ling, 1985; Wallace *et al.*, 1990) and the acetylation method was shown to be effective in the rumen (Wallace, 1992).

Table 4-3 summarises the results of Experiment 1 and Experiment 2. Although acetylation proved to be an effective way of delaying ruminal hydrolysis of β-casomorphins in this study, there are some hurdles to overcome before it can be applied to practical dietary supplementation of biologically active peptides. First, only 49.2, 51.8 and 35.8 % of the acetylated β-casomorphins would be expected to escape the ruminal degradation respectively if the breakdown rates are maintained *in vivo* (table 4-3). This degree of the protection, particularly for β-casomorphin-7, was far from complete and, hence, the escape rate of the acetylated peptides *in vivo* in various conditions should be evaluated fully before a successful development of rumen-stable delivery system for β-casomorphins can occur. Second, it is not known whether N-acetylated β-casomorphins escaping from the rumen can release active β-casomorphins in the small intestine. Since N-acetylated β-casomorphins are not expected to possess opioid activity (Henschen *et al.*, 1979), some form of modification of the acetylated peptides may be essential to ensure

Table 4-3. Half-life, breakdown rate and expected escape rate of N-acetylated and non-acetylated β-casomorphin.

	Experiment 1			Experiment 2					
	β-casomorphin			β-casomorphin			Acetyl-β-casomorphin		
	-4-NH_2	-5	-7	-4-NH_2	-5	-7	-4-NH_2	-5	-7
Half life[a], min	20.7	19.5	17.0	17.6	24.1	15.8	403	447	231
Breakdown rate[b]	0.24	0.26	0.29	0.28 (123)	0.21 (90)	0.31 (136)	0.01 (5.36)	0.01 (4.84)	0.02 (9.34)
Escape rate[c], %	4.7 (2.4)	4.5 (2.3)	3.9 (2.0)	4.1 (2.1)	5.5 (2.8)	3.7 (1.9)	49.2 (32.6)	51.8 (34.9)	35.8 (21.8)

[a] $t\frac{1}{2} = Log_n(2) /$ rate constant

[b] μmol / ml rumen liquor / h (nmol / mg of protein / h)

[c] Calculated from $P = \frac{C}{C+k}$ (Orskov, 1982), where P = proportion degraded, C = degradation rate (proportion h^{-1}) and k = outflow rate (proportion h^{-1}). Values are when $k = 0.1$ (0.05)

post-ruminal release of the peptides in active form. Acetylation of N-terminally extended β-casomorphins with an extension that is sensitive to pancreatic proteases would be one solution. Alternatively, a pH-sensitive polymeric coating might also meet the requirement (see Wu and Papas, 1997).

CHAPTER FIVE

THE EFFECT OF POST-RUMINAL INFUSIONS OF CASEIN AND β-CASOMORPHINS ON CIRCULATING GUT AND METABOLIC HORMONES IN DAIRY COWS

5.1. INTRODUCTION

It has been suggested that physiological actions of β-casomorphins in simple-stomached animals might occur via their influence on the circulating levels of some regulatory hormones such as insulin, prolactin and somatostatin as well as some gut hormones (e.g. CCK and PP) (see Chapter 1). Many studies have demonstrated that post-ruminal administration of casein increased milk production of lactating dairy cows (see section 1.3.2 for references). Furthermore, a number of studies reported that the post-ruminal casein infusions altered the partitioning of nutrients in favour of the mammary gland at the expense of adipose tissue. Such mobilisation or repartitioning of nutrients is regulated by metabolic hormones and gut hormones (Bauman and Currie, 1980; Martin *et al.*, 1993). Although some studies suggested that a possible mechanism underlying the superior milk production responses to post-ruminal casein over other proteins might be a repartitioning of nutrient use via changes in concentrations of metabolic hormones (Choung and Chamberlain, 1992c; Oldham, 1994), none of the studies closely monitored hormonal changes in response to post-ruminal casein infusion in dairy cows. Since β-casomorphins can be produced during the digestion of casein, it is possible that the positive milk production responses to post-ruminal casein derive, at least in part, from hormonal changes induced by β-casomorphins.

Therefore, two experiments were carried out to monitor hormonal changes to post-ruminal casein and β-casomorphins. The first experiment examined the effects of two levels of casein and an acid hydrolysate of casein infused intra-abomasally on circulating

levels of two major metabolic hormones (insulin and glucagon) and two gut hormones (GIP and GLP-1) in dairy cows. In the second experiment, three different levels of synthetic β-casomorphin mixtures were infused into the abomasum of lactating dairy cows to examine their effects on the levels of various hormones.

5.2. MATERIALS AND METHODS

5.2.1. Experiment 1

Animals

Three non-lactating Friesian cows of average body weight 531 kg, fitted with permanent ruminal cannulas, were used in Experiment 1. The animals were individually housed in metabolism stalls and fed 30 kg / d grass silage as a basal diet in two equal meals at 07:00 and 17:00 h. Water was available 24 hours a day. The chemical composition of the dietary ingredients is shown in table 5-1.

Experimental procedures

All animals received the basal diet for at least 7 days before the start of the experiment. On the day prior to the beginning of the experiment, abomasal infusion lines and jugular catheters were inserted at least 18 hours before the beginning of the infusion as described previously.

The experiment was designed as a duplicated 3 x 3 Latin square with infusion level fixed in each of the two blocks. Each block involved three animals, three treatments and three one-day periods. The experimental treatments for the first square were the basal diet alone (Con) and the basal diet plus abomasal infusions of 25 g / d of sodium caseinate (CSN1) or 27.5 g /d of an acid hydrolysate of casein (CAH1). The second square consisted of the same treatments but with the level increased to 50 (CSN2) and 55 (CAH2) g / d of the infusates respectively. Five days of resting time were given between the two blocks. All

Table 5-1. Chemical composition of silage used in Experiment 1.

Determinant	Content
Dry matter, g / kg	255
Organic matter, g / kg DM	898
pH	3.50
Total N, g / kg DM	16.8
True-protein N, g / kg N	285
NH_3-N, g / kg N	83
Water soluble carbohydrate, g / kg DM	55
Lactic acid, g / kg DM	80
Ethanol, g / kg DM	31
VFA	
Acetic acid	9
Propionic acid	0
Iso-butyric acid	0
Butyric acid	1
NDF, g / kg DM	625
ADF, g / kg DM	362

infusions within a block supplied equivalent amounts of all the amino acids; this was achieved by adding to CAH the acids destroyed during hydrolysis: 0.35 g of tryptophan, 1.49 g of glutamine and 0.83 g of asparagine to the 24.83 g / d of CAH1 (0.7, 2.98 and 1.66 g to the 49.66 g / d of CAH2 for the second block) to provide equivalent concentrations to those for CSN1 and CSN2. The infusates were dissolved in 500 ml of water and were infused at a rate of 30 ml / min using a peristaltic pump (Watson Marlow, Falmouth, UK). 500 ml of clean water was infused for the CON treatment. For the hormonal analysis, samples of blood were obtained from the jugular catheter at 09:30, 10:00, 10:30, 11:00, 11:30, 12:00, 13:00, 14:00, 15:00, 16:00 and 17:00 h on the day of infusions.

Statistical analysis

All results are presented as means of observations from 3 animals. Since REML analyses indicated that there was generally not a period effect and no carry-over effects were expected owing to the very short interval between the two squares, ANOVA was performed using Genstat 5 on the combined data from the two 3 x 3 Latin squares. The model used was: Y = Mean + Cow + Control + Control·Protein_Source + Control·Protein_Level + Control·Protein_Source·Protein_Level + Error. Comparisons between the control treatment and each individual treatment were performed by t-test using SED from the ANOVA and were considered significant when $P < 0.05$.

5.2.2. Experiment 2

Animals

Four lactating Friesian cows in weeks 29 to 34 of lactation, of average body weight 622 kg, fitted with permanent ruminal cannulas, were used in Experiment 2. The average milk yield of the cows during the experiment was 12.9 kg / d (milk fat, protein and lactose of 41, 35 and 45 g / kg respectively). The animals were individually housed in metabolism

stalls and allowed to become established on an ad libitum intake of silage plus 6 kg / d barley and 1 kg / d rapeseed meal as a basal diet. The animals were fed equal meals twice daily at 09:00 and 16:00 h and given free access to water. Consumption of concentrate was complete on all occasions and daily silage intakes varied little, ranging only from 34 to 37 kg / d throughout the experiment. The chemical composition of the dietary ingredients is shown in table 5-2.

Experimental procedures

All animals received the basal diet for at least 15 days before the start of the experiment. Abomasal infusion lines and jugular catheters were inserted as in Experiment 1. The animals were allocated according to a 4 x 4 Latin square design with four, three-day periods and four treatments. The experimental treatments were basal diet only (control) and basal diet plus three increasing amounts (120, 240 and 480 mg respectively) of abomasal infusions containing one-third by weight of each of three β-casomorphins (β-casomorphin-4-amide, β-casomorphin-5 and β-casomorphin-7). These dose levels were calculated based on published data (Brantl *et al.*, 1979) to be the amounts expected to be produced from 110, 220 and 440 g of casein respectively. The infusates were dissolved in 25 ml of water and were infused as a bolus using a 50 ml syringe. Blood samples were obtained from the jugular catheter at 08:45, 9:30, 10:00, 10:30, 11:00, 12:00, 13:00, 14:00 and 15:00 h on the day of infusions.

Statistical analysis

The results were subjected to ANOVA using Genstat 5 and comparisons between individual treatments were performed as for Experiment 1. The mean response of each

Table 5-2. Chemical composition of silage and concentrates used in Experiment 2.

Determinant	Silage	Barley	Rapeseed meal
Dry matter, g / kg	199	989	989
Organic matter, g / kg DM	909	977	919
pH	4.07	ND	ND
Total N, g / kg DM	22.8	17.9	58.9
True-protein N, g / kg N	247	ND	ND
NH_3-N, g / kg N	176	ND	ND
Water soluble carbohydrate, g / kg DM	9	ND	ND
Lactic acid, g / kg DM	100	ND	ND
Ethanol, g / kg DM	10	ND	ND
VFA			
Acetic acid	36	ND	ND
Propionic acid	6	ND	ND
Iso-butyric acid	0	ND	ND
Butyric acid	6	ND	ND
NDF, g / kg DM	582	331	391
ADF, g /kg DM	345	72	217
Starch, g / kg DM	ND	667	10
Sugar, g / kg DM	ND	15.4	87.0

ND: not determined

hormone was calculated by taking means of values for each time point corrected for any differences in basal values (i.e. values at -15 minutes).

5.3. RESULTS

5.3.1. Experiment 1

Plasma concentrations of insulin and glucagon, and the insulin / glucagon ratio

Concentrations of insulin and glucagon in plasma are shown in table 5-3 and 5-4 respectively. There were no statistically significant differences in the plasma insulin concentrations between the treatments. However, when the results were summarised in terms of responses over basal values, the insulin response during the 2 hours from the start of the infusions of the CSN treatments was lower than that of CAH at the higher levels of infusion ($P < 0.05$).

Plasma glucagon concentrations of CSN and CAH at both levels were significantly lower than CON at 6 hours after the infusions. However, for the daily mean glucagon concentration, only for the higher level of CSN was the value significantly lower than CON and the difference was not detected when the comparisons were made in terms of the responses over the control (table 5-8). A difference in plasma glucagon concentrations between the infusion levels of CSN was evident at 1 hour after the infusions and this difference was evident in the daily mean concentration. However, there were no differences between the treatments in the insulin / glucagon ratio (table 5-5).

Plasma concentrations of GIP and GLP-1

Table 5-6 and 5-7 respectively show the changes in GIP and GLP-1 concentrations in plasma during the sampling period. Only plasma GIP concentration at the higher level of CSN was significantly lower than the CAH treatment at 30 minutes after the infusion.

Table 5-3. Changes in concentrations (ng / ml) of insulin in blood plasma of non-lactating cows receiving a basal diet of grass silage (CON) with and without abomasal infusions of casein (CSN) or an acid hydrolysate of casein (CAH).

Time (min)	CON	CSN (g / d)		CAH (g / d)		SED				P value			
		25	50	27.5	55	C	L	S	LxS	C	L	S	LxS
-30	0.374	0.474	0.408	0.483	0.375	0.091	0.129	0.106	0.149	0.521	0.344	0.909	0.848
0	0.410	0.552	0.594	0.307	0.369	0.103	0.145	0.119	0.168	0.666	0.147	0.076	0.936
30	0.405	0.529	0.359	0.423	0.358	0.112	0.159	0.130	0.184	0.918	0.552	0.685	0.694
60	0.313	0.586	0.408	0.412	0.391	0.097	0.137	0.112	0.158	0.190	0.647	0.415	0.496
90	0.457	0.541	0.345	0.486	0.584	0.120	0.169	0.138	0.195	0.795	0.219	0.520	0.312
120	0.399	0.323	0.269	0.407	0.446	0.130	0.184	0.150	0.212	0.775	0.750	0.404	0.764
180	0.505	0.385	0.306	0.395	0.225	0.141	0.199	0.162	0.230	0.236	0.294	0.832	0.786
240	0.397	0.377	0.316	0.356	0.242	0.120	0.170	0.138	0.196	0.552	0.293	0.737	0.852
300	0.661	0.556	0.370	0.503	0.564	0.126	0.179	0.146	0.206	0.227	0.895	0.639	0.416
360	0.737	0.734	0.638	0.683	0.589	0.110	0.156	0.127	0.180	0.506	0.204	0.704	0.994
420	0.597	0.688	0.444	0.491	0.440	0.113	0.160	0.131	0.185	0.490	0.547	0.459	0.478
Daily Mean	0.485	0.462	0.405	0.450	0.417	0.074	0.105	0.086	0.121	0.501	0.249	0.995	0.890

Table 5-4. Changes in concentrations (pg / ml) of glucagon in blood plasma of non-lactating cows receiving a basal diet of grass silage (CON) with and without abomasal infusions of casein (CSN) or an acid hydrolysate of casein (CAH).

Time (min)	CON	CSN (g / d)		CAH (g / d)		SED				P value			
		25	50	27.5	55	C	L	S	LxS	C	L	S	LxS
-30	65.7	81.4	68.8	87.2	68.0	10.25	14.5	11.84	16.74	0.322	0.391	0.838	0.786
0	75.6	88.9	74.5	70.0	71.8	5.78	8.17	6.67	9.43	0.916	0.388	0.137	0.250
30	72.4	78.2	70.2	81.3	67.0	6.91	9.77	7.98	11.28	0.797	0.405	0.994	0.704
60	73.3^{ab}	103.1^{b}	60.0^{a}	91.8^{ab}	84.2^{ab}	10.72	15.16	12.38	17.51	0.310	0.117	0.615	0.181
90	79.7	82.8	68.9	74.0	84.6	6.74	9.53	7.78	11.00	0.764	0.444	0.666	0.147
120	82.0	75.7	72.3	71.9	74.1	8.92	12.62	10.31	14.57	0.362	0.742	0.924	0.792
180	89.7	88.6	63.9	72.7	77.5	16.99	24.02	19.61	27.74	0.428	0.840	0.954	0.470
240	81.8	68.1	64.3	78.5	69.8	13.34	18.86	15.40	21.78	0.404	0.278	0.618	0.877
300	94.0	94.5	64.8	73.9	74.8	10.02	14.16	11.57	16.36	0.119	0.452	0.657	0.215
360	104.4^{b}	74.3^{a}	70.7^{a}	72.7^{a}	82.5^{a}	4.57	6.46	5.27	7.46	<.001	0.510	0.359	0.230
420	93.1	76.0	60.3	78.9	80.5	10.30	14.57	11.89	16.82	0.092	0.838	0.353	0.485
Daily Mean	83.3^{b}	82.9^{b}	67.1^{a}	77.5^{ab}	75.9^{ab}	4.10	5.80	4.73	6.69	0.101	0.174	0.728	0.168

[a,b] Values within rows with different superscripts differ ($P < 0.05$).

Table 5-5. Changes in molar insulin / glucagon ratio of non-lactating cows receiving a basal diet of grass silage (CON) with and without abomasal infusions of casein (CSN) or an acid hydrolysate of casein (CAH).

Time (min)	CON	CSN (g / d)		CAH (g / d)		SED				P value			
		25	50	27.5	55	C	L	S	LxS	C	L	S	LxS
-30	4.20	4.26	3.84	4.30	6.37	1.484	2.099	1.714	2.424	0.749	0.825	0.470	0.485
0	4.11	4.52	5.17	2.86	4.18	1.013	1.433	1.170	1.655	0.942	0.278	0.284	0.785
30	4.19	4.33	3.79	3.77	5.72	1.369	1.936	1.581	2.235	0.878	0.652	0.674	0.448
60	3.51	4.37	5.75	3.91	2.84	1.240	1.754	1.432	2.025	0.581	0.871	0.267	0.412
90	4.09	4.15	4.17	4.80	4.75	0.983	1.390	1.135	1.605	0.710	0.190	0.601	0.977
120	3.09	3.41	3.25	3.79	4.26	0.949	1.343	1.096	1.550	0.549	0.862	0.540	0.780
180	3.42	3.40	4.73	3.85	2.45	0.999	1.413	1.153	1.631	0.859	0.465	0.446	0.265
240	3.62	4.34	5.35	3.72	3.21	1.058	1.496	1.221	1.727	0.622	0.820	0.285	0.547
300	4.91	4.33	4.98	4.90	7.37	1.292	1.828	1.492	2.110	0.716	0.536	0.344	0.556
360	4.58	6.54	8.19	6.83	5.08	1.461	2.067	1.687	2.386	0.185	0.843	0.422	0.337
420	4.08	6.94	6.13	3.96	3.45	1.478	2.090	1.707	2.414	0.499	0.876	0.129	0.933
Daily Mean	3.95	4.11	4.86	4.20	4.21	0.685	0.969	0.791	1.119	0.581	0.506	0.729	0.646

Table 5-6. Changes in concentrations (pg / ml) of GIP in blood plasma of non-lactating cows receiving a basal diet of grass silage (CON) with and without abomasal infusions of casein (CSN) or an acid hydrolysate of casein (CAH).

Time (min)	CON	CSN (g / d)		CAH (g / d)		SED				P value			
		25	50	27.5	55	C	L	S	LxS	C	L	S	LxS
-30	109.2^{ab}	138.0^{b}	58.0^{a}	80.7^{ab}	98.0^{ab}	18.2	25.8	21.0	29.7	0.415	0.159	0.689	0.043
0	90.9	126.0	110.7	94.7	96.1	17.1	24.2	19.8	28.0	0.409	0.759	0.275	0.682
30	124.6^{bc}	160.3^{c}	83.0^{a}	109.3^{ab}	135.6^{bc}	10.1	14.2	11.6	16.4	0.765	0.113	0.947	0.002
60	130.3	139.3	90.0	135.3	134.3	14.9	21.1	17.3	24.4	0.716	0.128	0.269	0.191
90	137.6^{ab}	110.3^{ab}	84.9^{a}	147.3^{b}	105.7^{ab}	15.1	21.4	17.5	24.7	0.124	0.205	0.136	0.655
120	126.3^{ab}	163.3^{b}	92.3^{a}	129.0^{ab}	103.3^{a}	16.5	23.3	19.0	26.9	0.798	0.036	0.553	0.260
180	95.4	103.3	79.7	122.3	76.3	19.0	26.9	22.0	31.1	0.856	0.327	0.730	0.624
240	95.7^{abc}	134.0^{bc}	88.3^{a}	139.3^{c}	89.6^{ab}	12.2	17.2	14.1	19.9	0.218	0.015	0.820	0.888
300	114.7^{ab}	96.7^{ab}	62.6^{a}	140.7^{b}	96.0^{ab}	15.6	22.1	18.0	25.5	0.342	0.146	0.060	0.775
360	133.0	97.0	76.0	125.0	92.0	24.1	34.1	27.8	39.4	0.168	0.641	0.441	0.843
420	136.0	119.0	102.0	128.0	75.0	26.8	37.9	30.9	43.7	0.271	0.275	0.776	0.580
Daily Mean	117.2^{ab}	126.3^{b}	84.3^{a}	123.3^{b}	97.7^{ab}	10.3	14.6	11.9	16.8	0.390	0.031	0.673	0.508

[a,b] Values within rows with different superscripts differ ($P < 0.05$).

Table 5-7. Changes in concentrations (pg / ml) of GLP-1 in blood plasma of non-lactating cows receiving a basal diet of grass silage (CON) with and without abomasal infusions of casein (CSN) or an acid hydrolysate of casein (CAH).

Time (min)	CON	CSN (g / d)		CAH (g / d)		SED				P value			
		25	50	27.5	55	C	L	S	LxS	C	L	S	LxS
-30	49.5	52.7	32.7	47.7	44.3	8.98	12.70	10.37	14.67	0.578	0.516	0.755	0.440
0	55.8	52.0	42.7	46.3	50.7	9.98	14.12	11.53	16.30	0.446	0.972	0.921	0.566
30	43.5	40.3	34.7	51.3	42.7	7.35	10.39	8.48	12.00	0.868	0.421	0.289	0.863
60	45.8	47.0	26.7	59.3	45.0	7.17	10.14	8.28	11.71	0.856	0.125	0.094	0.725
90	50.7^{ab}	51.7^{b}	31.3^{a}	47.0^{ab}	43.7^{ab}	5.51	7.79	6.36	8.99	0.217	0.121	0.560	0.211
120	49.3	44.0	32.7	36.7	48.3	9.31	13.16	10.75	15.20	0.361	0.891	0.706	0.310
180	56.5	31.7	34.7	46.0	49.3	9.15	12.94	10.56	14.94	0.109	0.937	0.200	0.988
240	51.7	40.3	52.3	40.0	33.7	11.10	15.70	12.82	18.13	0.385	0.965	0.476	0.491
300	51.8	49.3	60.0	52.0	49.3	12.07	17.07	13.94	19.71	0.946	0.809	0.780	0.643
360	61.0^{b}	55.0^{ab}	35.0^{a}	35.3^{a}	45.0^{ab}	6.15	8.70	7.10	10.04	0.013	0.549	0.512	0.063
420	60.3	38.0	34.7	49.3	50.3	7.26	10.26	8.38	11.85	0.039	0.703	0.138	0.801
Daily Mean	52.5	46.0	38.0	46.3	44.3	5.85	8.27	6.75	9.55	0.162	0.731	0.632	0.666

^{a,b} Values within rows with different superscripts differ ($P < 0.05$).

Table 5-8. Integrated responses[x] over the first 2 h and the whole 7 h respectively of insulin, glucagon, molar insulin / glucagon ratio, GIP and GLP-1 to intra-abomasal infusions of casein (CSN) or an acid hydrolysate of casein (CAH) in non-lactating cows receiving a basal diet of grass silage.

Item	Control	CSN (g / d)		CAH (g / d)		SED				P value			
		25	50	27.5	55	C	L	S	LxS	C	L	S	LxS
Insulin													
Mean120[y], pg / ml	2[ab]	-18[ab]	-156[a]	37[ab]	73[b]	58.2	82.3	67.2	95.1	0.767	0.464	0.061	0.226
Mean420[z], pg / ml	127	7	-112[†]	72	50	74.4	105.2	85.9	121.5	0.129	0.576	0.215	0.587
Glucagon													
Mean120, pg / ml	6.2	-0.2	-3.8	1.2	7.6	6.5	9.2	7.5	10.6	0.460	0.951	0.416	0.518
Mean420, pg / ml	17.0	-3.5	-5.6	-2.2	7.0	10.3	14.5	11.8	16.7	0.108	0.831	0.572	0.644
Insulin / Glucagon													
Mean120	-0.05	-0.32	1.40	0.48	-0.84	0.79	1.11	0.91	1.29	0.780	0.603	0.449	0.125
Mean420	0.21	0.30	0.68	0.90	-1.27	0.91	1.29	1.05	1.49	0.946	0.438	0.534	0.253
GIP													
Mean120, pg / ml	25.8	11.3	1.0	42.6	22.5	10.1	14.3	11.6	16.5	0.535	0.453	0.047	0.684
Mean420, pg / ml	7.1[ab]	-10.9[a]	-3.6[ab]	43.2[b]	-11.1[a]	13.8	19.5	16.0	22.6	0.848	0.345	0.175	0.082
GLP-1													
Mean120, pg / ml	-5.3	-6.6	-6.3	-1.6	-2.6	9.2	13.1	10.7	15.1	0.845	0.983	0.589	0.840
Mean420, pg / ml	0.7	-8.4	2.2	-1.8	-2.2	10.3	14.6	11.9	16.9	0.762	0.911	0.926	0.653

[x] The integrated response was calculated from the individual means after subtracting basal values in each animal.
[y,z] Integrated response between 30 and 120 minutes, and 30 and 420 minutes respectively after the start of infusions.
[a,b] Means within rows with different superscripts differ ($P < 0.05$).
[†] Values that tend to differ from control values ($P < 0.1$).

There were some indications of differences among the treatments at various time points as well as in the daily mean values. However, the variation in initial (-30 min) concentrations indicates a need for caution in interpretation of these differences. Even so, after allowance is made for the differences in values at -30 minutes, significant differences between the CSN and CAH treatments were still in evidence (table 5-8).

The pattern of the changes in plasma GLP-1 concentrations were very similar to those of glucagon except that the effects of CSN level appeared only at 90 minutes after the infusion and the inhibitory effect of the protein sources was less evident at 6 hours after the infusions. However, there were again no suggestions of differences between the treatments in the mean GLP-1 responses over control (table 5-8).

5.3.2. Experiment 2

Plasma concentrations of insulin and glucagon, and the insulin / glucagon ratio

There were no statistically significant differences between treatments in either the daily mean concentrations of plasma insulin or the insulin mean responses over the control (table 5-9). However, the insulin concentration with the highest level (480 mg) of the β-casomorphin infusion at 240 minutes was significantly lower than control (figure 5-1). Furthermore, there were various time points (30, 90, 240 minutes) where insulin concentrations with the β-casomorphin infusions tended ($0.05 < P < 0.1$) to be lower than control values (figure 5-1).

Figure 5-2 shows the changes of plasma glucagon concentrations during the sampling. Although there were no significant differences in daily mean values between the treatments, table 5-9 shows that the glucagon mean responses over control to the β-casomorphin infusions were linearly increased ($P < 0.05$) over both the first 3 and the whole 6 hours of the sampling period. There were also various time points where glucagon concentrations of the treatments tended to differ from corresponding control values as well

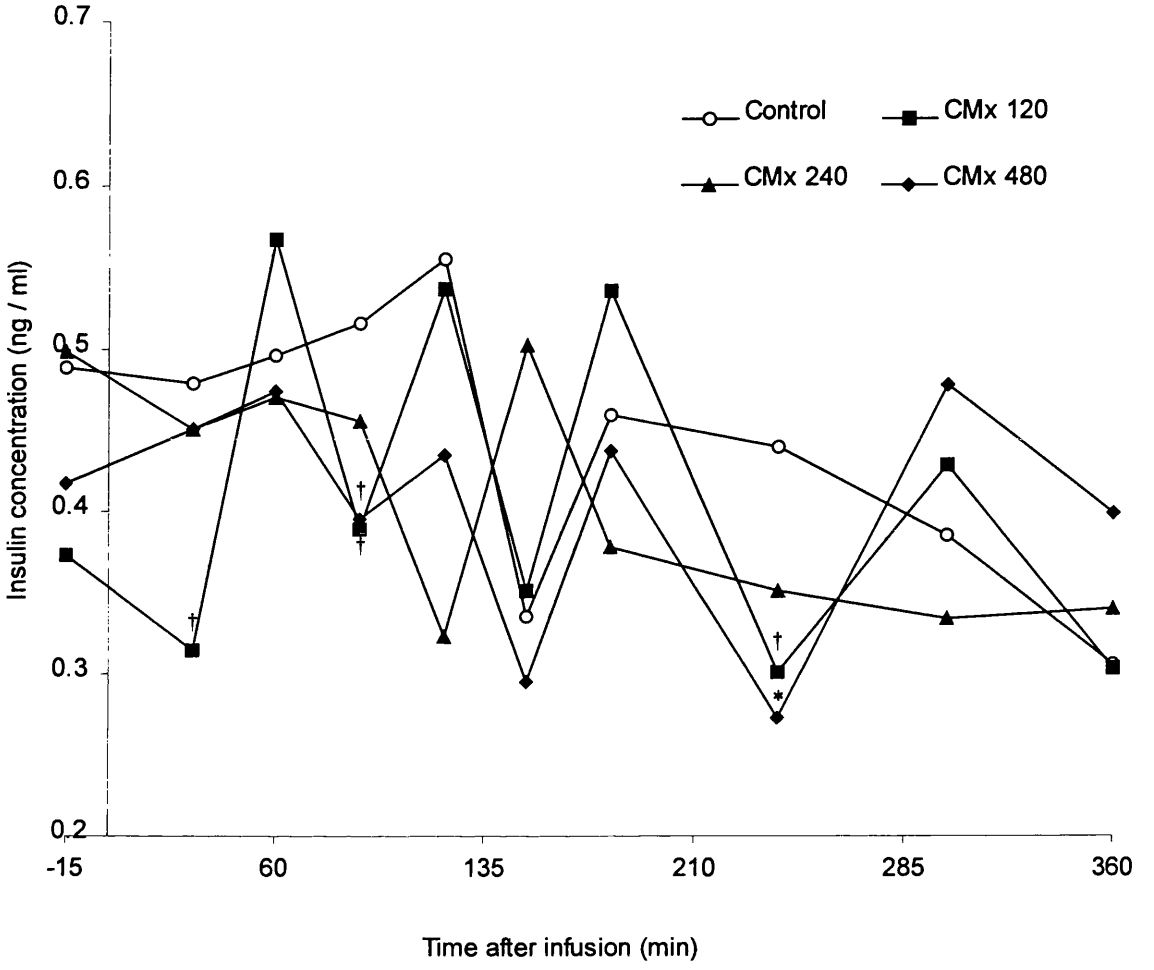


Figure 5-1. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma concentrations of insulin (ng / ml) in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked † when $P < 0.1$ and * when $P < 0.05$. Error bars are not indicated.

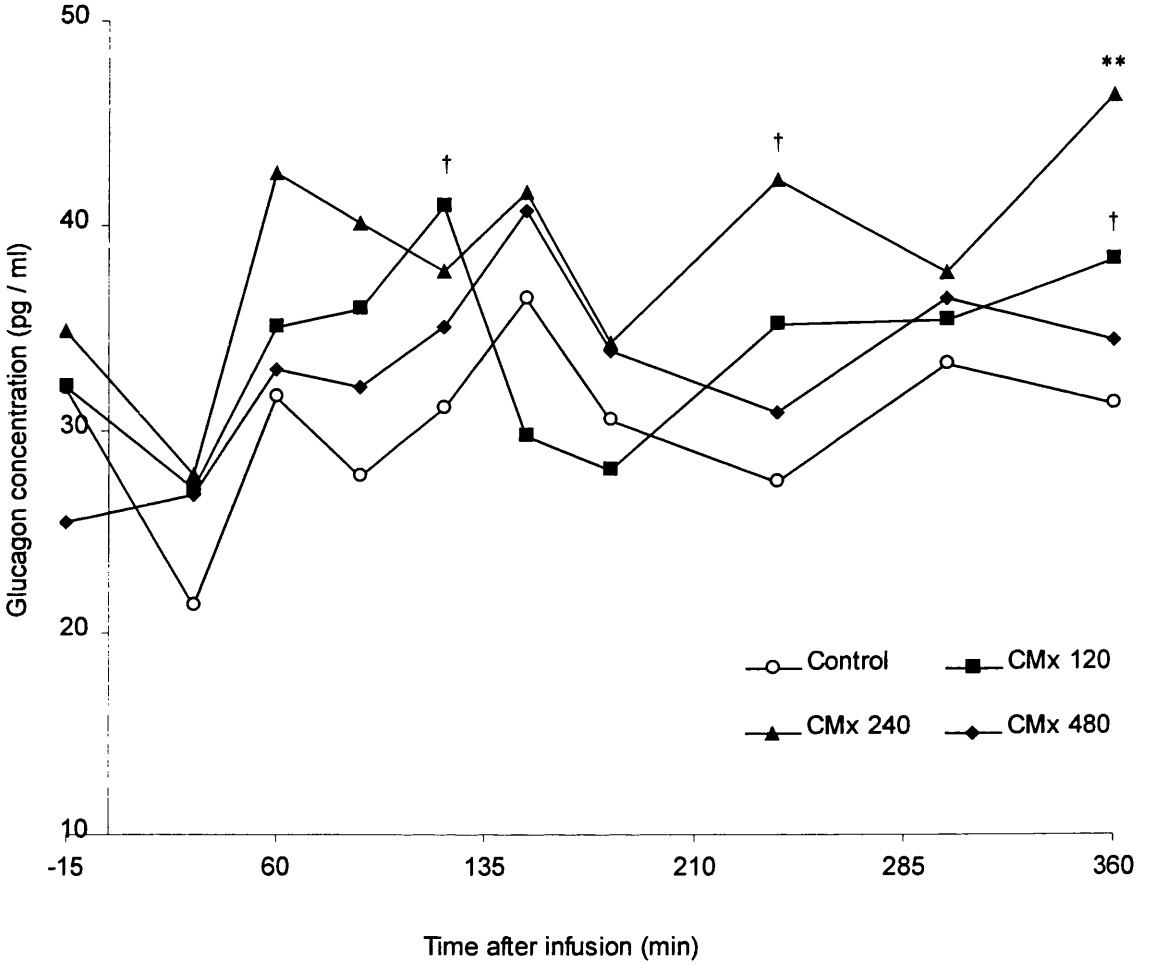


Figure 5-2. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma concentrations of glucagon (pg / ml) in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked † when $P < 0.1$ and ** when $P < 0.01$. Error bars are not indicated.

as a significantly different glucagon concentration of the mid-level infusion compared with control at 6 hours after the infusions. Overall, the glucagon response to β-casomorphin infusions was stimulatory in a dose dependent manner (table 5-9).

The changes in insulin / glucagon ratio during the sampling are given in figure 5-3. There were no effects of the β-casomorphin infusions on daily mean value or on the overall responses (table 5-9). However, all β-casomorphin infusions resulted in statistically significant decreases in the insulin / glucagon ratio at 4 hours compared with control.

Plasma concentrations of GIP and GLP-1

Figure 5-4 shows the changes in GIP concentrations in plasma during the sampling period. The daily mean GIP concentrations were significantly decreased by two levels (120 mg and 240 mg) of β-casomorphin mixture compared with control treatment (table 5-9) owing to the highly significant differences in the GIP concentrations between 2 and 3 hours after the infusions, there being a linear trend with level of β-casomorphin ($P < 0.05$). However, the GIP concentrations of β-casomorphin treatments before (-30 minutes) the infusions were all lower than that of control treatment, which might have affected the GIP concentrations later. Indeed, this is directly reflected in the result of the comparisons between the treatments in terms of the mean GIP response over control, shown in table 5-9, where no differences were found between the treatments.

Apart from some tendencies ($P < 0.1$) at some time points, there were no significant treatment effects on the plasma concentrations of GLP-1 (figure 5-5 and table 5-9).

Plasma concentrations of IGF-1 and GH

Plasma concentrations of GH (figure 5-6) and IGF-1 (figure 5-7) were little affected by the β-casomorphin infusions but both GH and IGF-1 concentrations at 90 minutes after

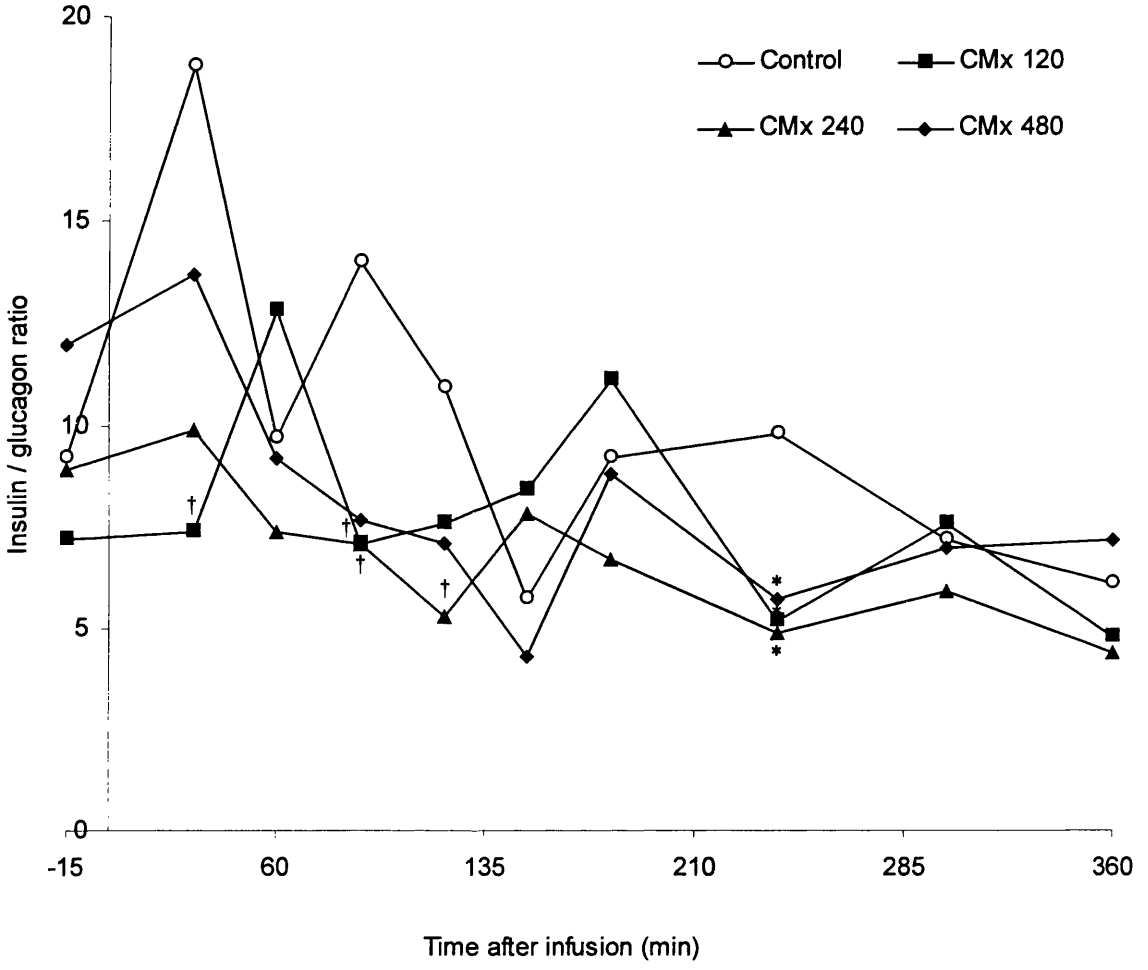


Figure 5-3. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma insulin / glucagon molar ratio in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked † when $P < 0.1$ and * when $P < 0.05$. Error bars are not indicated.

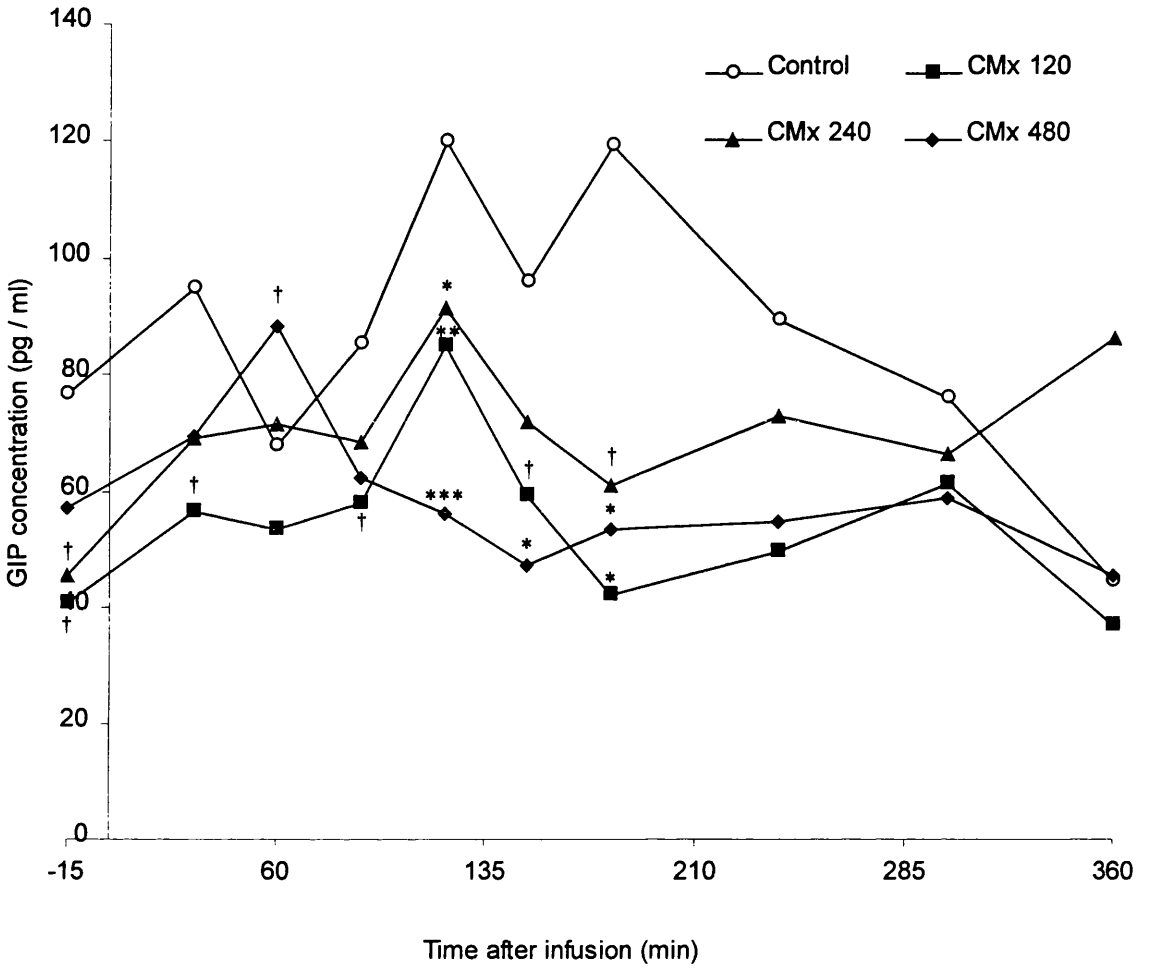


Figure 5-4. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma concentrations of GIP (pg / ml) in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked † when $P < 0.1$, * when $P < 0.05$, ** when $P < 0.01$ and *** when $P < 0.001$. Error bars are not indicated.

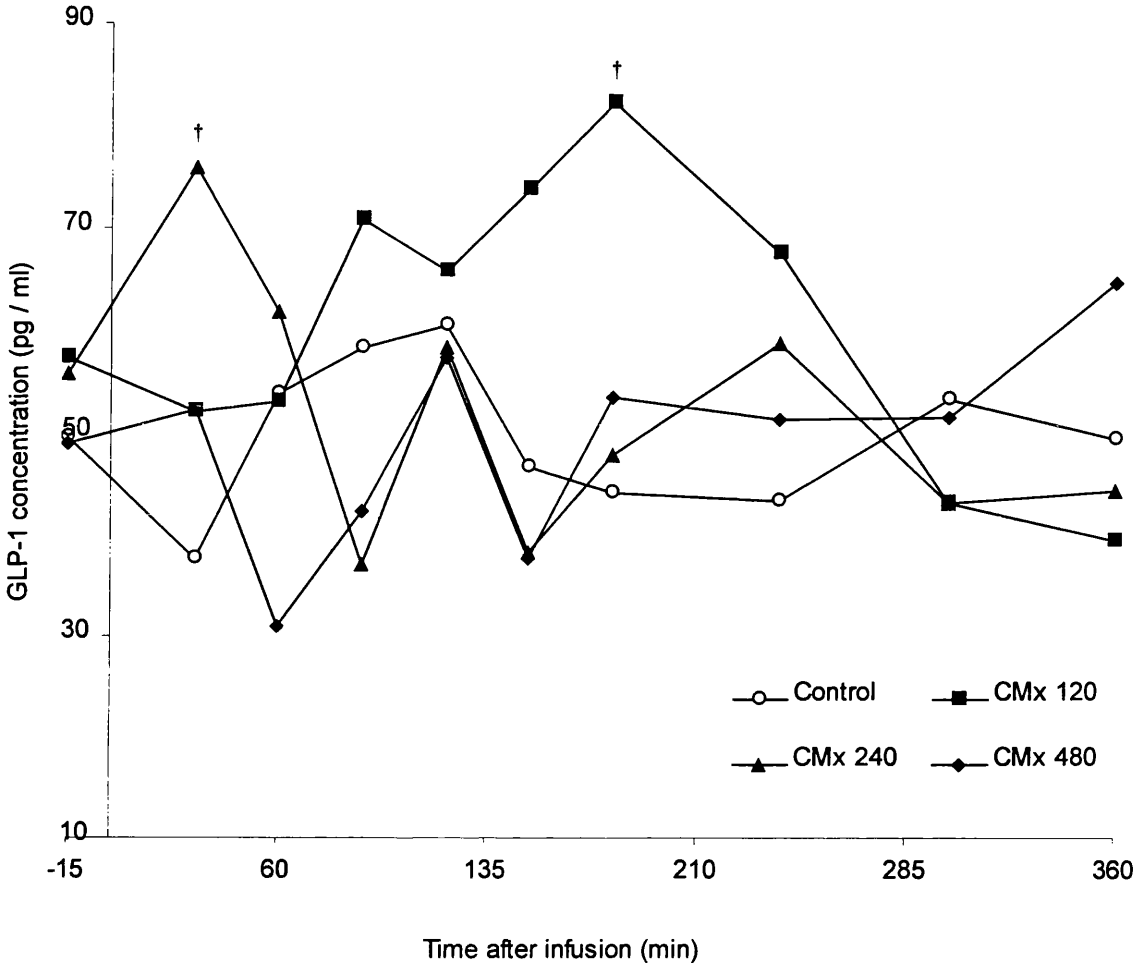


Figure 5-5. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma concentrations of GLP-1 (pg / ml) in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked † when $P < 0.1$. Error bars are not indicated.

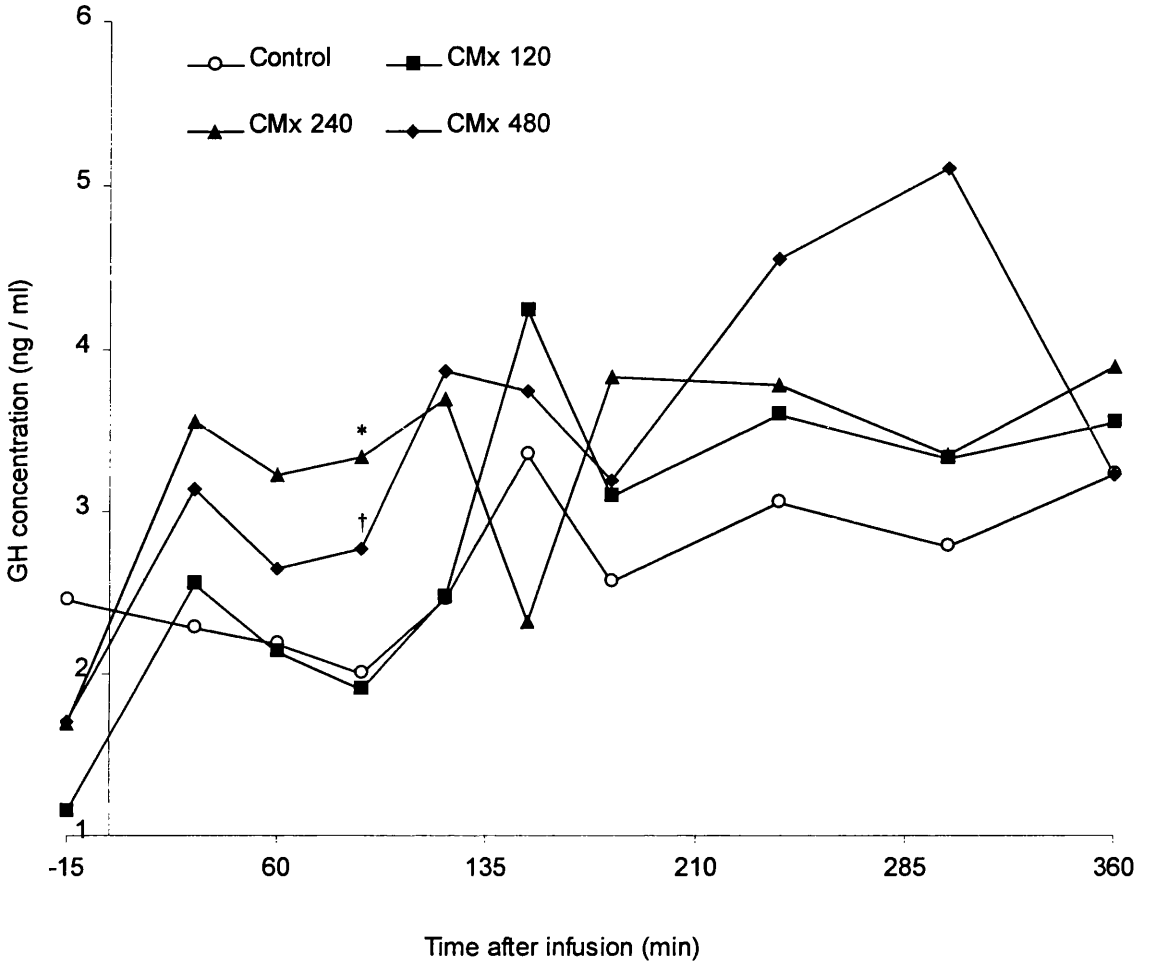


Figure 5-6. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma concentrations of GH (ng / ml) in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked † when $P < 0.1$ and * when $P < 0.05$. Error bars are not indicated.

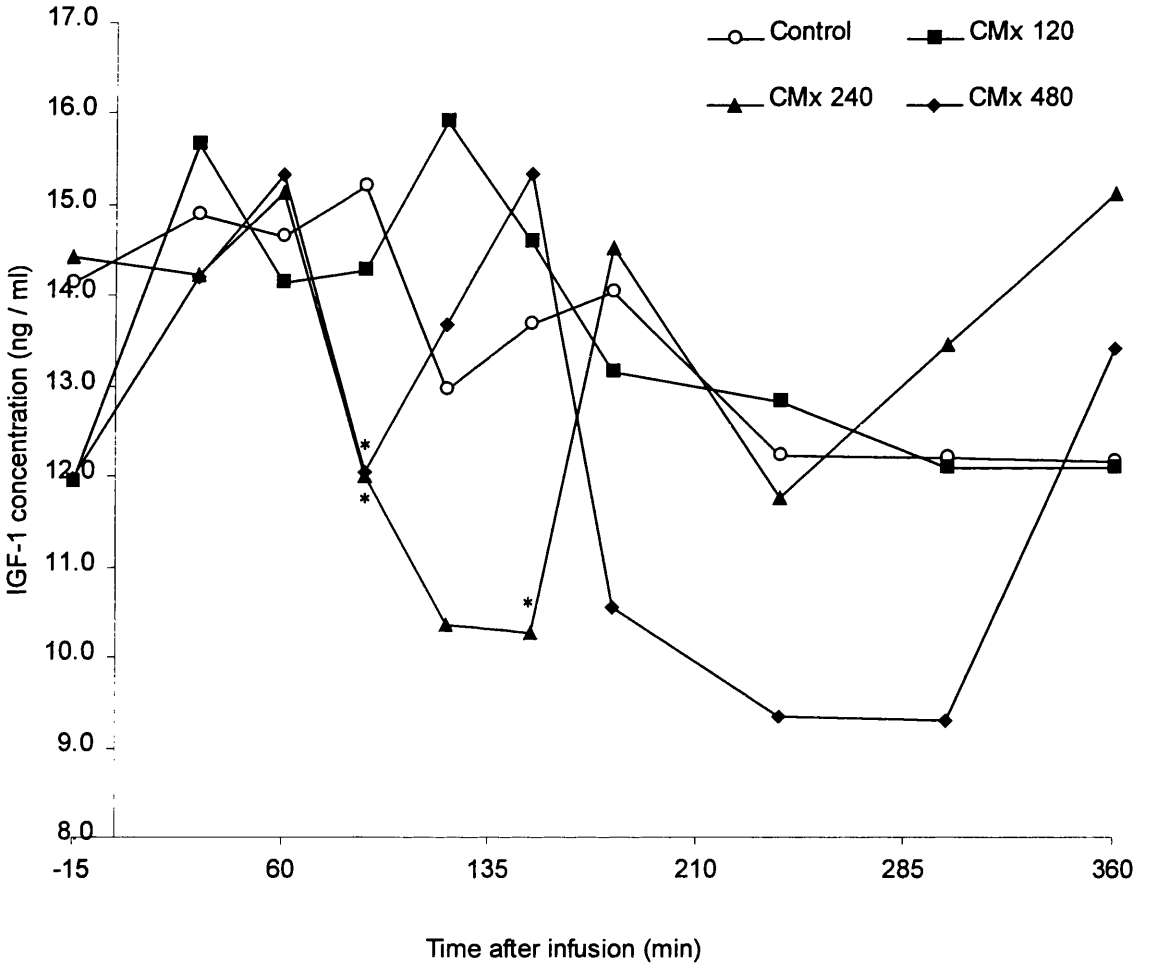


Figure 5-7. Effects of intra-abomasal infusions of three dose-levels (120, 240 and 480 mg) of β-casomorphin mixtures (CMx) on plasma concentrations of IGF-1 (ng / ml) in lactating dairy cows. Values are means of four cows. Values that differ from control values within individual times are marked * when $P < 0.05$. Error bars are not indicated.

Table 5-9. Daily mean and integrated responses[x] over the first 3 h and the whole sampling period respectively of insulin, glucagon, molar insulin / glucagon ratio, GIP, GLP-1, GH and IGF-1 to intra-abomasal infusions of increasing levels of β-casomorphin mixtures in lactating dairy cows.

Hormone	Control	β-Casomorphin mix.			SED	P value		
		120mg	240mg	480mg		Treat	Linear	Quad
Insulin								
Daily mean, pg / ml	446	410	410	405	63.9	0.911	0.604	0.700
Mean360[y], pg / ml	-56	33	-111	-17	75.2	0.358	0.968	0.691
Mean180[z], pg / ml	-14	75	-69	-3	100.3	0.586	0.800	0.886
Glucagon								
Daily mean, pg / ml	30.3	33.7	38.5	32.9	4.91	0.464	0.628	0.174
Mean360, pg / ml	-1.7[a]	2.0[ab]	4.4[ab]	8.2[b]	3.50	0.128	0.028	0.662
Mean180, pg / ml	-2.1[a]	0.7[ab]	2.5[ab]	8.1[b]	3.51	0.119	0.025	0.919
Insulin / Glucagon								
Daily mean	9.0	7.5	6.5	7.5	1.31	0.379	0.329	0.163
Mean360	0.5	0.5	-2.6	-4.4	3.05	0.373	0.116	1.000
Mean180	2.2	1.9	-1.5	-3.5	3.46	0.368	0.111	0.935
GIP								
Daily mean, pg / ml	86.9[a]	54.3[b]	69.7[ab]	58.5[b]	9.11	0.043	0.061	0.167
Mean360, pg / ml	9.7	13.7	27.2	1.2	14.9	0.431	0.586	0.179
Mean180, pg / ml	20.3	18.3	26.8	5.8	13.7	0.524	0.343	0.365
GLP-1								
Daily mean, pg / ml	49.5	60.5	52.0	49.0	10.9	0.711	0.715	0.515
Mean360, pg / ml	-0.4	2.6	-4.7	1.2	15.5	0.966	0.993	0.830
Mean180, pg / ml	0.6	9.0	-2.6	-3.4	16.4	0.868	0.658	0.852
GH								
Daily mean, ng / ml	2.99	2.80	3.26	3.39	0.49	0.640	0.318	0.898
Mean360, ng / ml	0.27	1.94	1.79	2.04	1.22	0.482	0.270	0.388
Mean180, ng / ml	0.02	1.59	1.62	1.51	1.17	0.500	0.331	0.301
IGF-1								
Daily mean, ng / ml	13.63	13.68	13.13	12.52	1.12	0.722	0.303	0.868
Mean360, ng / ml	-0.86	1.60	-1.35	0.01	2.10	0.555	0.999	0.936
Mean180, ng / ml	0.08	2.69	-1.67	1.55	2.35	0.361	0.861	0.616

[x] The integrated response was calculated from the individual means after subtracting basal values (values at -15 minutes) in each animal.
[y] Integrated response between 30 and 360 minutes after the infusions.
[z] Integrated response between 30 and 180 minutes after the infusions.
[a,b] Means within rows with different superscripts differ ($P < 0.05$).

the infusions were significantly ($P < 0.05$) and linearly affected by the β-casomorphin infusions.

5.4. DISCUSSION

In both experiments, variation in the initial concentrations of some hormones, especially GIP, made the interpretation of the results difficult. A possible explanation of the initial differences may be the lack of control over the pattern of eating during the experiment although the overall intake was relatively constant. In the case of GIP, since the RIA method used in the experiment was an optimised method for sheep plasma samples, analytical insensitivity coupled with very low circulating concentrations of GIP in lactating animals may have affected the reliability of the measurement.

Nevertheless, although the emerging picture from the results of Experiment 1 was somewhat complicated, there were clear suggestions of differences in responses of some hormones, notably insulin and GIP, to the post-ruminal administrations of the casein and the hydrolysate. Both infusates contained an equal amount of total amino acids in which only the potential yield of peptides during digestion was different (only approximately 15 % of amino acids were in peptide form in the CAH treatments, based on amino acid analysis before and after complete hydrolysis in 6 M HCl in this laboratory). Amino acids in peptide form are known to be taken up more rapidly in the small intestine than those in free amino acid form since peptides are transported by a single high capacity carrier with a broad specificity while free amino acids compete with each other for their specific carriers (Webb and Matthews, 1998). Therefore, the differences in the responses of some hormones to casein and its acid hydrolysate could derive from differences in rates of absorption in the small intestine. The significantly different responses of glucagon and GLP-1 to both CSN

and CAH infusions at 6 hours compared with CON may be indications that the rate of amino acid absorption following the infusions may have contributed, at least in part, to the changes in some of the circulating hormones.

Another possible explanation of the differences between the two infusates is the involvement of β-casomorphins because the potential for production of β-casomorphins during post-ruminal digestion is considered negligible for CAH. On this basis, furthermore, the higher level of CSN would be expected to produce more β-casomorphins than the other treatments. In this connection, it is interesting to note that there were particularly marked responses of insulin and GIP to the higher levels of CSN. Effects of opioids on insulin secretion have been reported in simple-stomached animals (see below).

In Experiment 2, the three infusion levels were calculated based on results of Brantl *et al.* (1979) and were equivalent to the amount expected to be produced from approximately 110, 220 and 440 g of casein respectively which are very similar to the levels of infusion used in the experiments of Choung and Chamberlain (1992c). The mixture of the three β-casomorphins used in the experiment was assumed to be representative of bioactive peptides produced during the digestion of casein since β-casomorphin-5 and -7 were reported to be the two principal products of the digestion of casein in the study of Brantl *et al.* (1979) and β-casomorphin-4-amide best describes the characteristics (μ-ligand specific opioids) of the bioactivity of β-casomorphins (Henschen *et al.*, 1979).

There are a number of reports on the actions of exorphins and endogenous opioids on pancreatic endocrine responses. In general, the responses of circulating pancreatic hormones to the exorphins are stimulatory although some reports are contradictory. For example, both oral and intravenous β-casomorphins have been reported to augment insulin secretion that is prestimulated by amino acids and glucose infusions in dogs, but intravenous infusions of β-casomorphins failed to alter basal insulin secretion

(Schusdziarra *et al.*, 1983a; Schusdziarra *et al.*, 1983d). Furthermore, oral and intravenous gluten exorphins have also been shown to increase postprandial insulin and glucagon levels in rats (Fukudome *et al.*, 1995) while there was no response of insulin to gluten exorphins in a peptic hydrolysate of gluten in humans (Morley *et al.*, 1983). These results suggest that there may be species differences in the responses of pancreatic hormones to exogenous opioids and also that metabolic status (e.g. blood glucose concentration) may be an important determinant of hormonal responses to opiates (Schusdziarra *et al.*, 1984).

In the results of Experiment 2, insulin did not respond to the increasing amounts of β-casomorphins. Insulin concentration is known to be lower and to be less sensitive to exogenous glucose in lactating animals compared with non-lactating or pregnant animals (Satin *et al.*, 1985; Faulkner and Martin, 1999). Therefore, apart from the species difference mentioned above, the lack of insulin response to the β-casomorphins might have been because the animals used in the experiment were lactating. However, there were some suggestions of differences in insulin concentrations at individual time points and the directions of the differences were consistently inhibitory which is consistent with an involvement of β-casomorphins in Experiment 1.

Glucagon, by contrast, was stimulated linearly by the infusions of the β-casomorphin mixtures ($P < 0.05$). This result is in line with an observation that glucagon concentration was significantly higher after post-ruminal administrations of casein compared with an acid hydrolysate of casein similar to that used here (Choung and Chamberlain, 1998). The stimulation of glucagon seen in Experiment 2 is also supported by other studies where administrations of exorphins and endogenous opioids stimulated glucagon in simple-stomached animals (Kanter *et al.*, 1980; Ipp *et al.*, 1982; Fukudome *et al.*, 1995). Since it is expected that the stimulation of glucagon secretion would induce an increment in the rates of hepatic gluconeogenesis and glycogenolysis, availability of glucose would be increased, thus providing an adequate supply for lactose synthesis and

milk production. Indeed, the opioid system has been reported to stimulate glucagon secretion, resulting in increased blood glucose level in men (Johansen *et al.*, 1992). In addition, although there was little difference in the insulin patterns among the treatments, the significant increases in the glucagon concentration led to a suggestion of linear trends in effects on the insulin / glucagon molar ratios, in terms of the integrated responses, which might also have a physiological importance in the alteration of hepatic gluconeogenesis and nutrient partitioning during early lactation (Unger, 1972; Bassett, 1975).

There is very little information available on the influences of the opioid system on secretion of the other hormones monitored in this study. However, GH has been reported to be stimulated by administration of morphine and DAMME (D-Ala^2-MePhe^4-Met-enkephalin-(O)-ol), a typical μ-ligand opiate (Delitala *et al.*, 1984). This may explain the early stimulation of GH by the two higher levels of β-casomorphin observed in Experiment 2. One possible explanation for the lack of the response in some hormones in Experiment 2 may be the comparatively low dose levels of β-casomorphins used in the experiment since some of the studies where clear hormonal responses by exorphins were demonstrated in simple stomached animals used dosage levels that were more than 100 to 1000 times higher (30 to 300 mg / kg body weight) than the dosage used in Experiment 2 (approximately 0.2 to 0.8 mg / kg body weight).

Identifying possible underlying mechanisms from the results of the two experiments is not easy especially since responses of some hormones to the casein and the β-casomorphin infusions were not clearly demonstrated. However, most effects of the casein and the β-casomorphin infusions seem to be compatible with an involvement of somatostatin. Somatostatin is known to play a vital role in regulating pancreatic secretion (see Yamada and Chiba, 1989; Chiba and Yamada, 1994 for review). It has been shown to inhibit insulin secretion through receptor linkage to a G_i protein (Fehmann and Habener, 1992) and such inhibition has been demonstrated *in vivo* in humans (D'Alessio *et al.*,

1989) and in sheep (Rose *et al.*, 1996; Martin and Faulkner, 1996). Support for an involvement of somatostatin in the actions of β-casomorphins comes from reports of a stimulation of somatostatin or SLI (somatostatin-like imunoreactivity) concentrations in simple-stomached animals by exorphins including β-casomorphins (e.g. Morley *et al.*, 1983; Schusdziarra *et al.*, 1983b). Furthermore, β-casomorphins have been reported to bind to somatostatin receptors in human mammary cells (Hatzoglou *et al.*, 1996), and endogenous opioids have been shown to play a role in the control of the arginine vasopressin response to insulin-induced hypoglycemia by interacting with somatostatin in man (Chiodera and Coiro, 1991). Therefore, it is reasonable to hypothesise that the inhibitory responses of insulin and GIP in these experiments may derive from interactions between β-casomorphins and somatostatin.

CHAPTER SIX

FURTHER INVESTIGATIONS OF THE EFFECT OF POST-RUMINAL INFUSION OF β-CASOMORPHINS ON INSULIN AND GLUCOSE LEVELS IN DAIRY COWS AND THE POSSIBLE INVOLVEMENT OF SOMATOSTATIN-28

6.1. INTRODUCTION

In the results of experiments in Chapter 5, there were clear indications of effects of post-ruminal casein and β-casomorphins on pancreatic hormones and, as discussed in that chapter, these changes could be mediated via somatostatin.

There are two major molecular forms of somatostatin derived from the prosomatostatin molecule. Somatostatin-28 (SS-28), which is predominantly produced by and distributed in the small intestine, responds to intake of food, particularly fat and protein (Chiba and Yamada, 1994) whereas somatostatin-14 is little affected by food intake (Ensinck *et al.*, 1990; Greenberg, 1993). It is therefore believed that SS-28 plays an important endocrine role in the control of metabolic hormones such as insulin in response to food ingestion and, indeed, inhibition of insulin secretion by SS-28 has been demonstrated both in simple-stomached animals (D'Alessio *et al.*, 1989) and in ruminant animals (Martin and Faulkner, 1996).

In the present chapter, as a further investigation of the role of β-casomorphins in the control of pancreatic hormones, four experiments were carried out. Experiment 1 was designed to identify the action of β-casomorphins on the secretion of insulin induced by a post-ruminal glucose infusion and to determine whether their action is compatible with an involvement of SS-28. Because of concern about twice daily feeding in the previous chapter, frequent feeding using an automatic feeder was introduced to lessen the effects of feeding on hormonal changes.

As the inhibitory action of β-casomorphins on insulin secretion was clearly demonstrated in non-lactating dairy cows in Experiment 1, the next step, in Experiment 2b, was to determine whether β-casomorphins would exert a similar action on the secretion of insulin in lactating cows. In addition, an attempt was also made to determine whether the results from previous experiments were due to the presence of the 'unnatural' β-casomorphin-4-amide in the infused mixture of β-casomorphins. Prior to the main experiment, Experiment 2a was carried out with lactating dairy cows as a preliminary experiment to ascertain optimum experimental conditions for the main experiment.

Finally, Experiment 3 was carried out to confirm the result of Experiment 1, this time with intravenous glucose in order to maintain sustained levels of plasma insulin, and to investigate a possible importance of the levels of glycaemia in the response of insulin to the β-casomorphins.

6.2. MATERIALS AND METHODS

6.2.1. Experiment 1

Animals

Four non-lactating Friesian cows of average body weight 527 kg, fitted with permanent ruminal cannulas, were used in Experiment 1. The animals were individually housed in metabolism stalls and fed 30 kg / d grass silage plus 2 kg / d barley as a basal diet in eight equal meals at 07:00, 10:00, 13:00, 16:00, 19:00, 22:00, 01:00 and 03:00 h using an automatic feeder. Water was available 24 hours a day. The chemical composition of the silage used in the experiment is shown in table 6-1.

Table 6-1. Chemical composition of silage and concentrates used in Experiments 1 and 3.

Determinant	Experiment 1		Experiment 3	
	Silage	Barley	Silage	Sugar beet cubes
Dry matter, g / kg	221	855	227	852
Organic matter, g / kg DM	914	977	918	883
pH	3.66	ND	3.96	ND
Total N, g / kg DM	22.3	18.5	26.3	18.4
True-protein N, g / kg N	217	ND	175	ND
NH_3-N, g / kg N	138	ND	157	ND
Water soluble carbohydrate, g / kg DM	8	ND	21	ND
Lactic acid, g / kg DM	124	ND	72	ND
Ethanol, g / kg DM	13	ND	3	ND
VFA				
Acetic acid	27	ND	24	ND
Propionic acid	0	ND	1	ND
Iso-butyric acid	0	ND	0	ND
Butyric acid	0	ND	0	ND
NDF, g / kg DM	541	292	514	350
ADF, g /kg DM	347	56	341	217
Starch, g / kg DM	ND	679	ND	6
Sugar, g / kg DM	ND	18	ND	226

ND: not determined

Experimental procedures

At least 7 days were given to all animals to adapt to the experimental environment before the start of the experiment. On the day prior to the beginning of the experiment, abomasal infusion lines and jugular catheters were inserted at least 18 hours before the beginning of the infusion as described previously.

The experiment was designed as a 4 x 4 non-randomised block with a fixed treatment sequence, but individual cows did not receive any given treatments on the same day. Each animal served as its own control. The experimental treatments in the order in which they were applied were the basal diet alone (Control) and the basal diet plus abomasal infusion of 100 g of glucose dissolved in 1 L of distilled water at a rate of 6.67 g glucose / min (Glucose) with and without either intravenous infusion of SS-28 at a rate of 52 pg / kg BW / min (Glucose + SS) or abomasal infusion of a bolus of 240 mg β-casomorphin mixture containing 80 mg of each of the three β-casomorphins (Glucose + CMx). The glucose was infused for 15 minutes using a peristaltic pump starting at 16:00 h and the SS-28 was infused for 15 minutes using a syringe pump at 16:00 h. The β-casomorphins were dissolved in 25 ml of water and were infused as a bolus using a 50 ml syringe at 16:00 h. For the analysis of insulin and glucose, samples of blood were obtained from the jugular catheter at 15-minute intervals from 15:15 to 19:00 h.

Statistical analysis

Since no period effect was expected owing to the very short periods (total experimental period of no longer than 13 days for each animal) period effect was ignored. The data were summarised as integrated responses during the first 90 and 180 minutes, and ANOVA was performed using Genstat 5 on the summary to determine statistically significant effects of treatment. Data are given as means with SED and differences were deemed statistically significant when $P < 0.05$.

6.2.2. Experiment 2a

Animals

Three lactating Friesian cows in weeks 12 to 17 of lactation, of average body weight 557 kg, fitted with permanent ruminal cannulas, were used in Experiment 2a. The animals were individually housed in metabolism stalls and offered 60 kg / d silage plus 6 kg / d barley as a basal diet. The animals were fed equal meals twice daily at 08:00 and 15:00 h and given free access to water. During a 14-day adaptation period silage intakes were recorded and the amount offered to individual animals (ranging between 45 and 50 kg / d) was adjusted in order to make the consumption complete. Consumption of the concentrate was complete on all occasions throughout the experiment. The chemical composition of the dietary ingredients is shown in table 6-2.

Experimental procedures

All animals received the basal diet for at least 14 days before the start of the experiment. Abomasal infusion lines and jugular catheters were inserted as in Experiment 1 at least 24 hours before the start of the infusions. The animals received two treatments as a simple replicated design with three-day periods. The experimental treatments were basal diet only (control) and basal diet plus 200 g / d abomasal glucose (glucose). The glucose was dissolved in 1 L of distilled water and was infused at a rate of 13.3 g glucose / min starting at 15:00 h. Blood samples were obtained from the jugular catheter at 15-minute intervals from 14:15 to 18:00 h.

Statistical analysis

The data were summarised as for Experiment 1 except that only one integrated response during the period between 15 and 180 minutes was used. The results were

subjected to ANOVA using Genstat 5 and comparisons between the treatments were performed as for Experiment 1.

6.2.3. Experiment 2b

Animals

Four lactating Friesian cows in weeks 20 to 26 of lactation, of average body weight 568 kg, fitted with permanent ruminal cannulas, were used in Experiment 2b. The animals were housed and fed as for Experiment 2a. The chemical composition of the dietary ingredients is shown in table 6-2.

Experimental procedures

All experimental procedures were as for Experiment 2a except that there were two more treatments in addition to Control and Glucose. They were basal diet plus the abomasal glucose with either 240 mg of the mixture of three β-casomorphins (Glucose + CMx) or 80 mg of β-casomorphin-4-amide (Glucose + CM4a). The animals were allocated according to a 4 x 4 Latin square design with four, three-day periods and the four treatments. Blood samples were obtained from the jugular catheter as for Experiment 2a.

Statistical analysis

The results were subjected to ANOVA using Genstat 5 and comparisons between individual treatments were performed on summarised data as for Experiment 1 except that integrated responses were divided into three phases, during the periods between 15 and 60, 75 and 180 and 15 and 180 minutes.

Table 6-2. Chemical composition of silage and concentrates used in Experiments 2a and 2b.

Determinant	Silage		Barley
	Exp. 2a	Exp. 2b	
Dry matter, g / kg	247	239	841
Organic matter, g / kg DM	924	921	978
pH	3.48	3.60	ND
Total N, g / kg DM	17.7	19.7	19.4
True-protein N, g / kg N	212	214	ND
NH_3-N, g / kg N	127	123	ND
Water soluble carbohydrate, g / kg DM	8	8	ND
Lactic acid, g / kg DM	124	126	ND
Ethanol, g / kg DM	13	12	ND
VFA			
Acetic acid	20	16	ND
Propionic acid	1	0	ND
Iso-butyric acid	0	0	ND
Butyric acid	1	1	ND
NDF, g / kg DM	564	542	236
ADF, g /kg DM	364	467	57
Starch, g / kg DM	ND	ND	622
Sugar, g / kg DM	ND	ND	19

ND: not determined

6.2.4. Experiment 3

Animals

Five non-lactating Friesian cows of average body weight 579 kg, fitted with permanent ruminal cannulas, were used in Experiment 3. The animals were individually housed in metabolism stalls and fed 20 kg / d grass silage plus 5 kg / d sugar beet cubes as a basal diet in eight equal meals using an automatic feeder as for Experiment 1. Water was available 24 hours a day. The chemical composition of the silage used in the experiment is shown in table 6-1.

Experimental procedures

At least 14 days were given to all animals to adapt to the experimental environment before the start of the experiment. On the day prior to the beginning of the experiment, abomasal infusion lines and jugular catheters were inserted at least 24 hours before the beginning of the infusion as described previously.

The animals were allocated according to a 5 x 5 Latin square design with five, 3-day periods and five treatments. The experimental treatments were the basal diet alone (Control) and the basal diet plus bolus intravenous infusion of glucose (36 mg / kg BW) followed by continuous intravenous infusion of glucose at a rate of 1 mg / kg BW / min (G I) with and without an abomasal bolus infusion of 240 mg β-casomorphin mixture containing 80 mg of each of the three β-casomorphins (G I + CMx) and the basal diet plus bolus intravenous infusion of glucose (72 mg / kg BW) followed by continuous intravenous infusion of glucose at a rate of 2 mg / kg BW / min (G II) with and without the abomasal infusion of the β-casomorphin mixture (G II + CMx). For the bolus glucose infusion, the glucose was dissolved in 30 ml saline and infused at 16:00 h using a 50 ml syringe. For the continuous infusion treatments, glucose was infused using a syringe pump starting at 16:15 h for 3 hours and the β-casomorphins were injected as in Experiment 1 at

16:00 h. Since only one jugular vein was used, continuous glucose infusion was stopped every 15 minutes, starting at 10 minutes after the start of the infusions, for 5 minutes in which time samples of blood were obtained from the jugular catheter for the analysis of insulin and glucose as in Experiment 1.

Statistical analysis

The data were summarised as integrated responses as for Experiment 1 and subjected to ANOVA using Genstat 5.

6.3. RESULTS

6.3.1. Experiment 1

The results of Experiment 1 are shown in figure 6-1 and 6-2 and summarised in table 6-3. Figure 6-1 shows the changes of plasma insulin concentrations during the sampling period. Insulin responded quickly to abomasal glucose starting to rise from just 15 minutes after the infusion and reached its peak between 30 and 45 minutes, after which the insulin concentration started to decrease gradually to the starting values. The integrated response of insulin during the first 90 minutes to the infusion of glucose was significantly reduced when β-casomorphin or SS-28 was co-administered but the effect was not significant when total responses were compared (table 6-3).

The glucose concentrations of all treatments with the glucose infusion were significantly higher than that of Control but there were no statistically significant differences between the treatments (table 6-3).

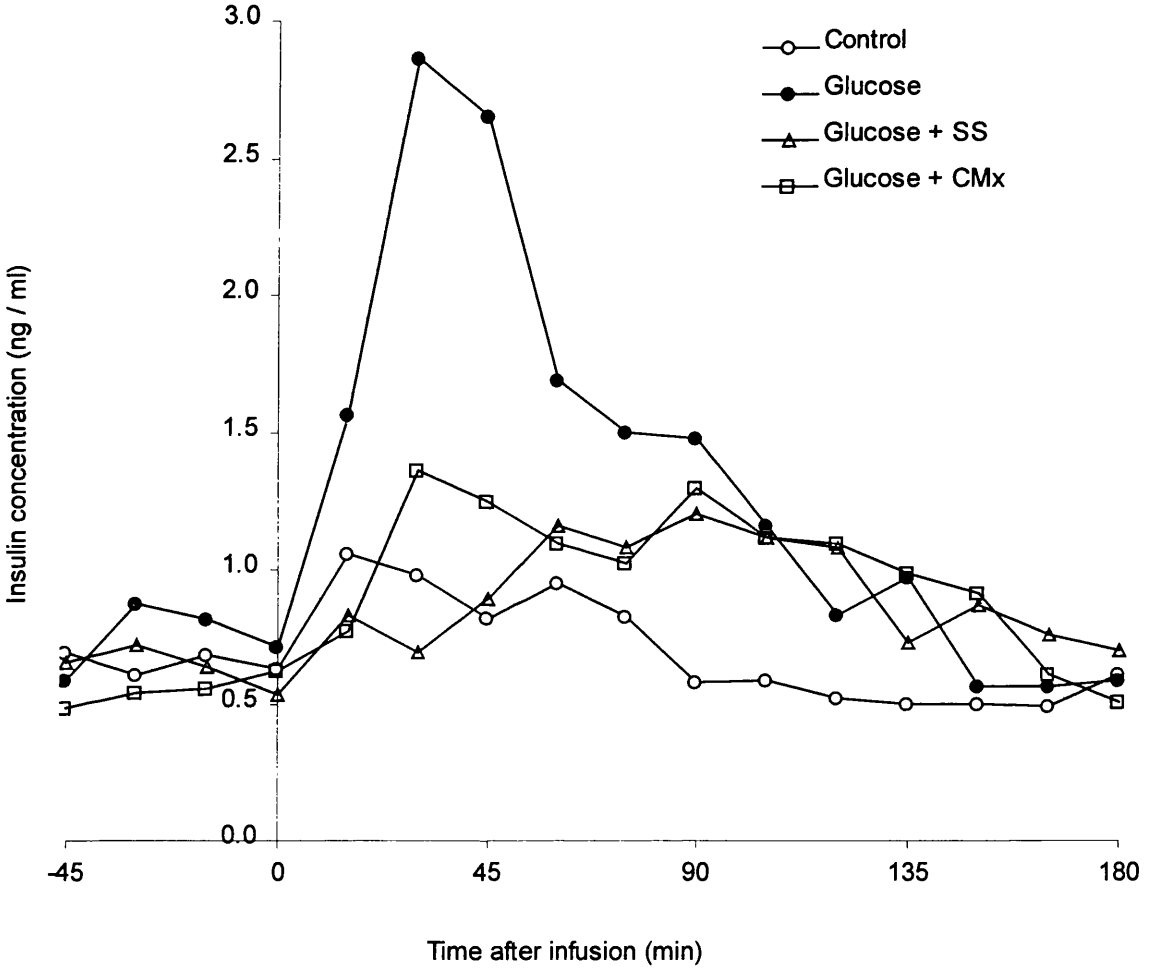


Figure 6-1. Effects of intra-abomasal infusions of glucose, glucose + a mixture of β-casomorphins (glucose + CMx) and glucose + intravenous somatostatin-28 (glucose + SS) on plasma concentrations of insulin (ng / ml) in non-lactating dairy cows. Values are means of four cows. For clarity SEM is not indicated.

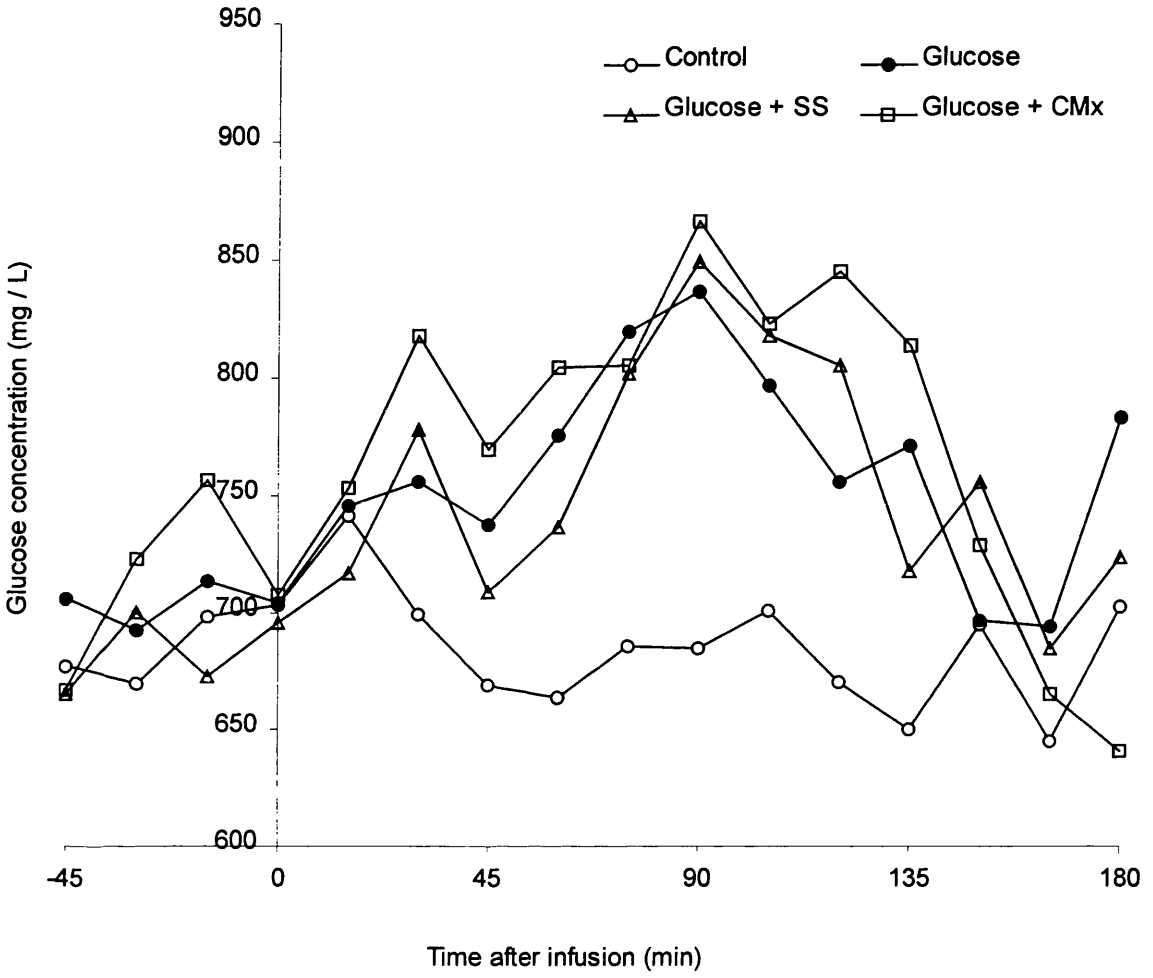


Figure 6-2. Effects of intra-abomasal infusions of glucose, glucose + a mixture of β-casomorphin (glucose + CMx) and glucose + intravenous somatostatin-28 (glucose + SS) on plasma concentrations of glucose (mg / L) in non-lactating dairy cows. Values are means of four cows. For clarity SEM is not indicated.

Table 6-3. Integrated responses[x] of insulin and glucose to intra-abomasal infusions of glucose with and without intra-abomasal β-casomorphin mixtures or intravenous somatostatin-28 in non-lactating dairy cows.

Item	Control	------ Intra-abomasal glucose ------			SED
		alone	plus somatostatin-28	plus β-casomorphin	
Insulin					
Response[y], ng / ml	0.211[a]	1.208[b]	0.337[a]	0.576[a]	0.242
Total[z], ng / ml	0.046[a]	0.621[b]	0.286[ab]	0.446[b]	0.151
Glucose					
Response[y], mg / L	-9[a]	70[b]	91[b]	93[b]	31
Total[z], mg / L	-3[a]	60[ab]	74[b]	64[ab]	30

[x] The integrated response was calculated from the individual means after subtracting basal values (means of values between -45 and 0 minutes) in each animal.

[y] Integrated response between 15 and 90 minutes for insulin, 30 and 135 for glucose.

[z] Integrated response between 15 and 180 minutes.

[a,b] Means within rows with different superscripts differ ($P < 0.05$).

6.3.2. Experiment 2a

Changes in plasma concentrations of insulin and glucose in response to the abomasal glucose infusion are shown in figure 6-3 and 6-4 respectively. The insulin concentration rose progressively just 15 minutes after the infusion and reached its peak at approximately 90 minutes. However, there was no significant difference between the treatments in the patterns of change in glucose concentrations, although the glucose concentrations in response to the glucose infusion appeared to be higher compared with Control during the period between 45 and 105 minutes. The failure to reach significance in glucose response was only due to one cow that did not show any difference between the treatments. Table 6-4 shows the difference in the integrated responses of insulin and glucose to the glucose infusion in which only the insulin response was significantly increased by the glucose infusion.

6.3.3. Experiment 2b

Changes in the patterns of plasma insulin secretion and glucose concentration are shown in figure 6-5 and 6-6 respectively. Table 6-5 summarises the results in terms of the integrated responses during the time periods of 15 to 60, 75 to 180 and 15 to 180 minutes.

In the integrated responses of glucose during all time periods and of insulin during the time periods of 75 to 180 and 15 to 180 minutes, all treatments with glucose infusions resulted in significantly higher insulin and glucose concentrations compared with the control treatment but there were no differences among the treatments with the glucose infusion. However, the integrated response of insulin to Glucose during the period of initial increase (15 to 60 minutes) was not significantly different from Control whereas the responses to both Glucose + CMx and Glucose + CM4a were significantly higher than Control. Over this period, the insulin response to the additional infusion of β-casomorphin-

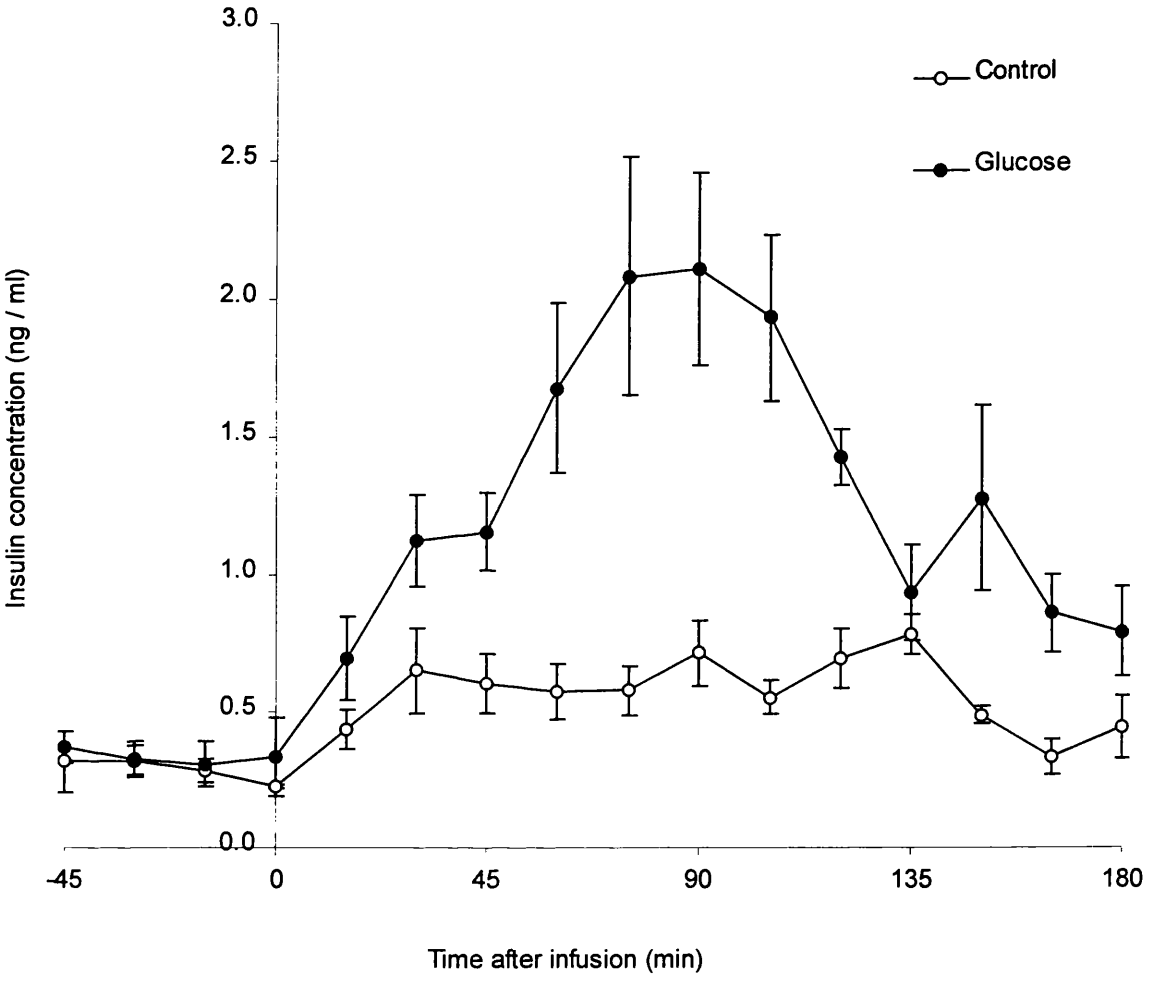


Figure 6-3. Effects of intra-abomasal infusions of glucose on plasma concentrations of insulin (ng / ml) in lactating dairy cows. Values are means of three cows with vertical bars indicating SEM.

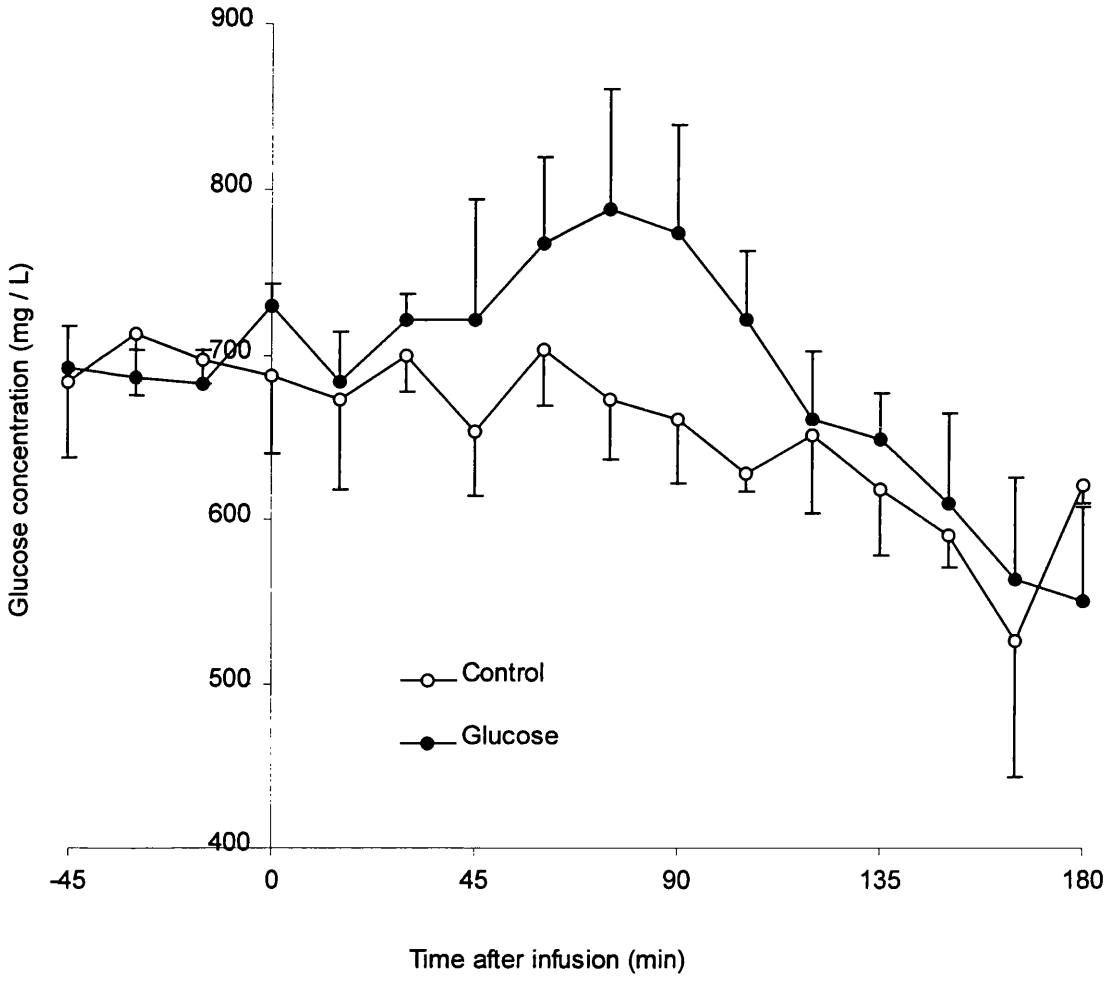


Figure 6-4. Effects of intra-abomasal infusion of glucose on plasma concentrations of glucose (mg / L) in lactating dairy cows. Values are means of three cows with vertical bars indicating SEM.

Table 6-4. Integrated responses* of insulin and glucose to intra-abomasal infusions of glucose in lactating dairy cows.

Item	Control	Glucose	SED	P value
Insulin, ng / ml	0.29	1.00	0.08	0.013
Glucose, mg / L	-56.1	16.3	40.2	0.214

* The integrated response was calculated from the individual values after subtracting basal values (means of values between -45 and 0 minutes) in each animal and taking means over the sampling period between 15 and 180 minutes.

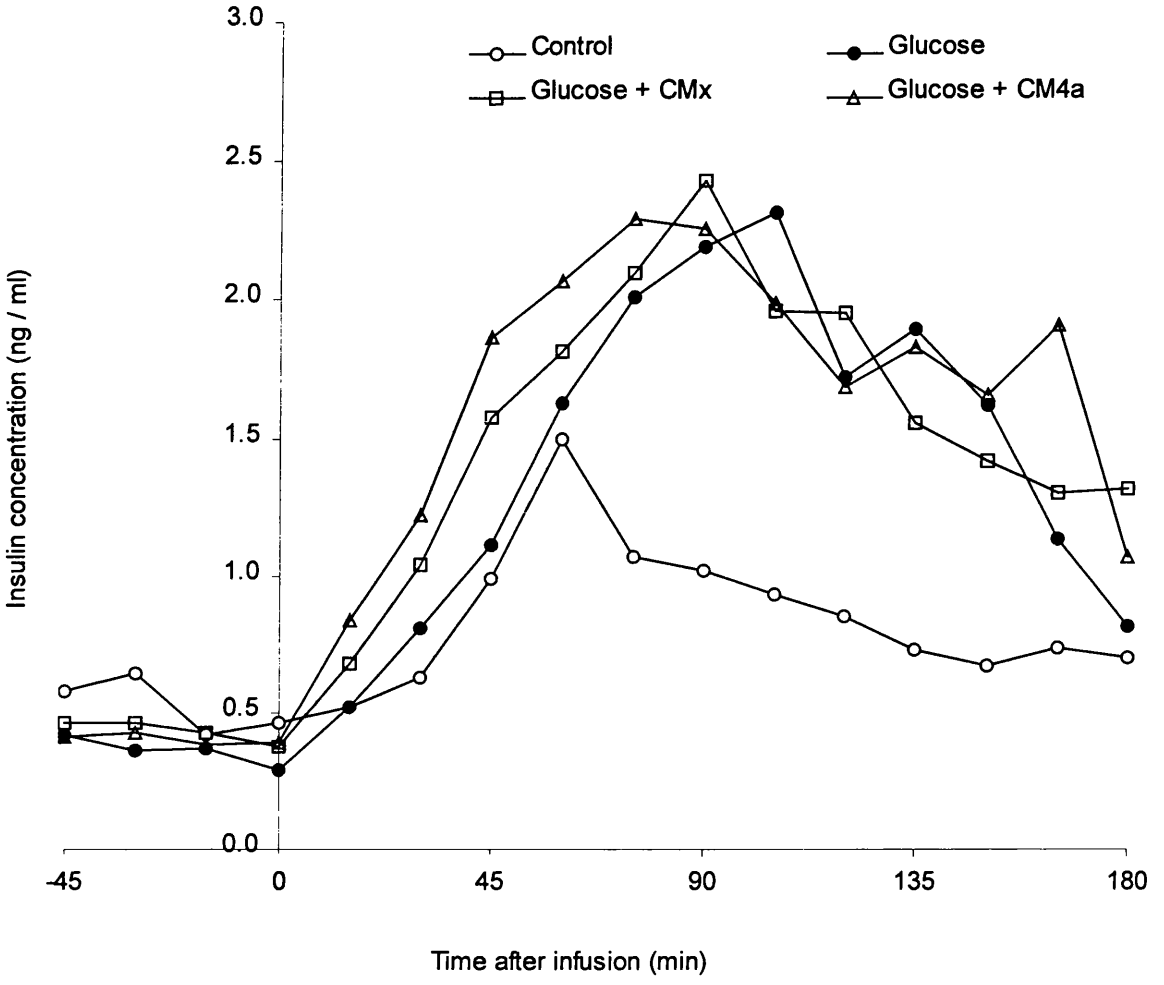


Figure 6-5. Effects of intra-abomasal infusions of glucose with and without a mixture of β-casomorphins (glucose + CMx) or β-casomorphin-4-amide (glucose + CM4a) on plasma concentrations of insulin (ng / ml) in lactating dairy cows. Values are means of four cows. For clarity SEM is not indicated.

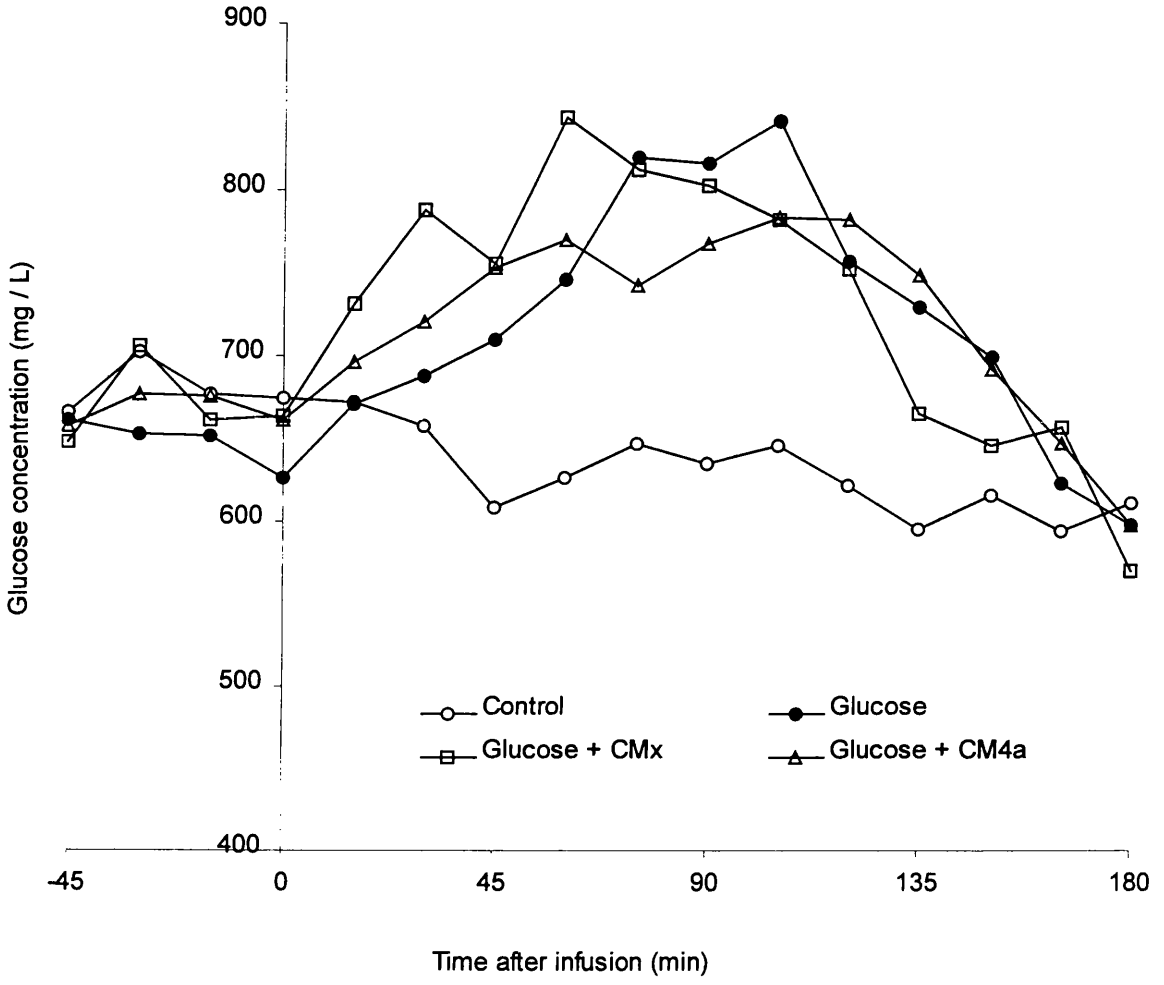


Figure 6-6. Effects of intra-abomasal infusions of glucose with and without a mixture of β-casomorphins (glucose + CMx) or β-casomorphin-4-amide (glucose + CM4a) on plasma concentrations of glucose (mg / L) in lactating dairy cows. Values are means of four cows. For clarity SEM is not indicated.

Table 6-5. Integrated responses* of insulin and glucose to intra-abomasal infusions of glucose with and without intra-abomasal infusion of a mixture of β-casomorphins or β-casomorphin-4-amide in lactating dairy cows.

Item	Control	Intra-abomasal glucose			SED
		alone	plus β-casomorphin mixture	plus β-casomorphin -4-amide	
Insulin					
15-60 min, ng / ml	0.38[a]	0.65[ab]	0.85[bc]	1.10[c]	0.178
75-180 min, ng / ml	0.32[a]	1.35[b]	1.32[b]	1.43[b]	0.314
15-180 min, ng / ml	0.34[a]	1.12[b]	1.16[b]	1.32[b]	0.228
Glucose					
15-60 min, mg / L	-37[a]	50[b]	103[b]	55[b]	31.9
75-180 min, mg / L	-57[a]	79[b]	43[b]	56[b]	38.5
15-180 min, mg / L	-50[a]	69[b]	63[b]	55[b]	33.9

* The integrated response was calculated from the individual means after subtracting basal values (means of values between -45 and 0 minutes) in each animal during the given time periods.

[a,b,c] Means within rows with different superscripts differ ($P < 0.05$).

4-amide, but not β-casomorphin mixture, was significantly higher than to the infusion of glucose alone.

6.3.4. Experiment 3

Figure 6-7 and 6-8 show the results of insulin and glucose respectively and table 6-6 summarises the results. There were rapid and proportionate increases in plasma concentrations of insulin and glucose in response to the two levels of intravenous glucose infusions. During the first 90 minutes, the insulin response to the higher level of glucose infusion was significantly inhibited by the concomitant intra-abomasal infusion of the β-casomorphin mixture but the inhibitory action was not evident when the lower level of glucose was infused (table 6-6).

The integrated responses of plasma glucose to the intravenous glucose infusions were clearly increased proportionately but the differences in the responses to the different glucose levels were less evident compared with the insulin response. There was no effect of the β-casomorphins at either level of glucose infusion although the plasma glucose responses to the glucose infusions were generally higher when accompanied by the abomasal β-casomorphin mixture compared with those to the glucose alone especially during the later period (90 to 180 minutes).

6.4. DISCUSSION

The results of Experiment 1 clearly demonstrate that post-ruminal administration of the β-casomorphin mixture can significantly suppress the insulin rise in response to post-ruminal glucose infusion. Furthermore, the inhibitory action of the β-casomorphin is compatible with the action of SS-28 as judged from its effects on the insulin secretion when administered intravenously. The insulinopenic action of β-casomorphin is further

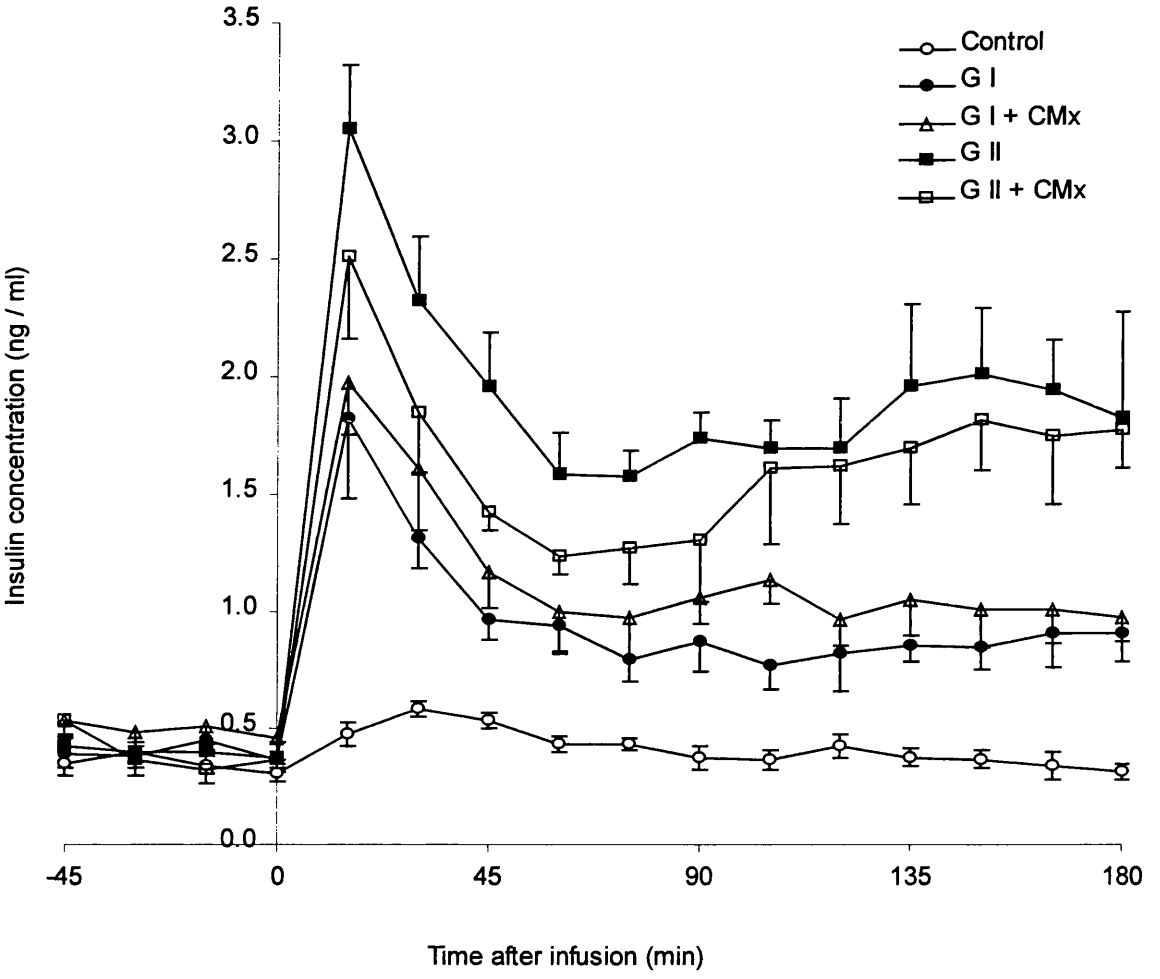


Figure 6-7. Effects of intra-abomasal infusions of a mixture of β-casomorphins (CMx) on plasma concentrations of insulin (ng / ml) prestimulated by two levels of intravenous glucose (G I and G II) in non-lactating dairy cows. Values are means of five cows with vertical bars indicating SEM.

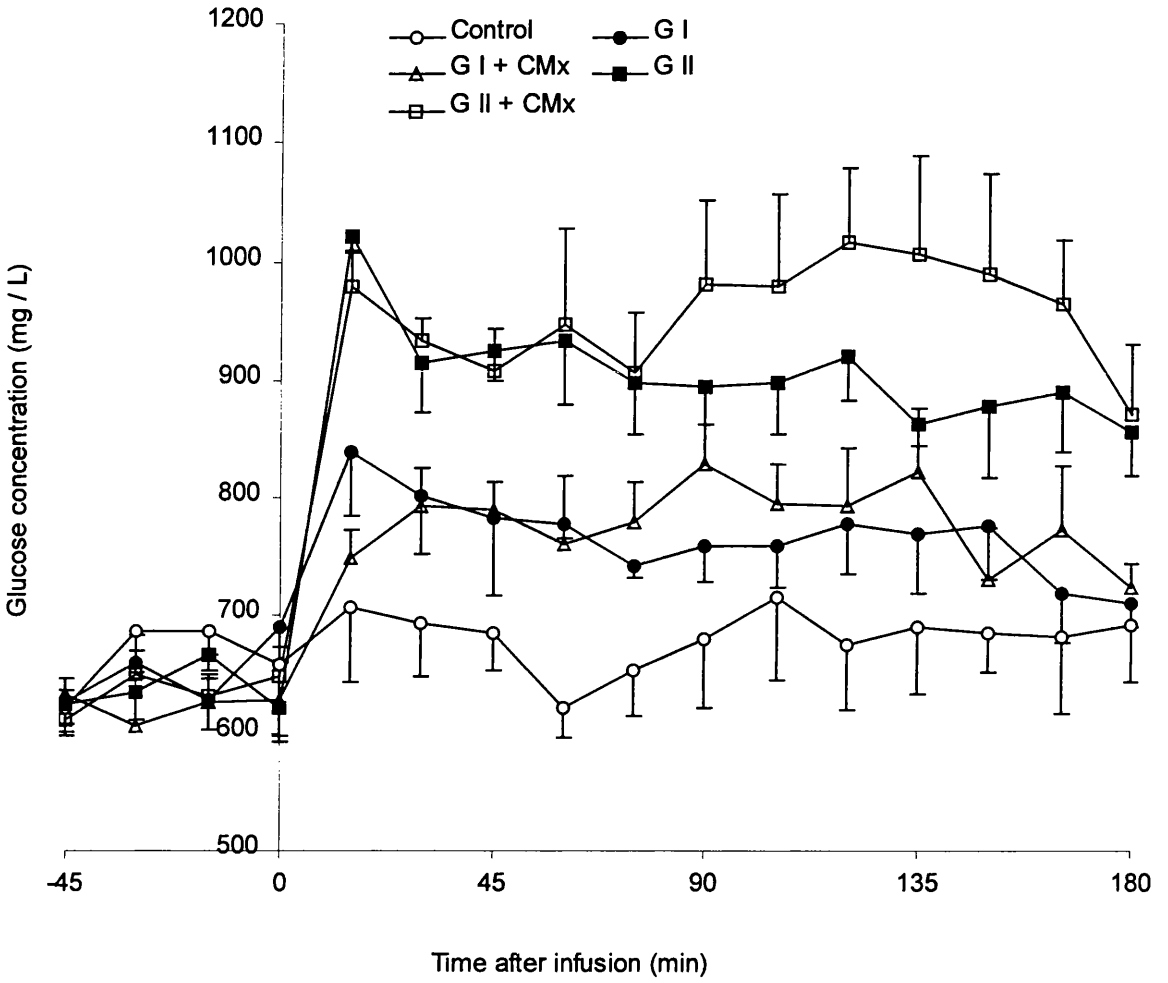


Figure 6-8. Effects of intra-abomasal infusions of a mixture of β-casomorphins (CMx) on plasma concentrations of glucose (mg / L) prestimulated by two levels of intravenous glucose (G I and G II) in non-lactating dairy cows. Values are means of five cows with vertical bars indicating SEM.

Table 6-6. Integrated responses* of insulin and glucose to two levels of intra-abomasal infusions of glucose with and without intra-abomasal β-casomorphin mixtures (β-CMx) in non-lactating dairy cows.

Item	Control	----- Glucose I -----		----- Glucose II -----		SED
		alone	plus β-CMx	alone	plus β-CMx	
Insulin						
15-90, ng / ml	0.12[a]	0.72[b]	0.80[b]	1.64[d]	1.20[c]	0.147
90-180, ng / ml	0.02[a]	0.46[b]	0.53[b]	1.44[c]	1.25[c]	0.154
15-180, ng / ml	0.07[a]	0.59[b]	0.67[b]	1.55[c]	1.26[c†]	0.140
Glucose						
15-90, mg / L	18[a]	133[b]	162[b]	297[c]	310[c]	38.9
90-180, mg / L	27[a]	99[a]	154[ab]	257[bc]	348[c]	55.4
15-180, mg / L	24[a]	116[ab]	153[b]	277[c]	327[c]	42.9

* The integrated response was calculated from the individual means after subtracting basal values (means of values between -45 and 0 minutes) in each animal during the given time periods.

[a,b,c,d] Means within rows with different superscripts differ ($P < 0.05$).

† Tend to differ from Glucose II alone ($0.05 < P < 0.1$)

confirmed by the results of Experiment 3 where insulin was prestimulated by a combination of bolus and continuous intravenous infusion of glucose. These results are in agreement with the results for insulin in Chapter 5 in which inhibition of insulin concentration by post-ruminal casein and β-casomorphin mixtures was indicated.

Although there are a limited number of reports available, previously reported actions of β-casomorphin on insulin are mainly stimulatory. In dogs, both oral (Schusdziarra *et al.*, 1983d) and intravenous (Schusdziarra *et al.*, 1983a) administrations of various β-casomorphins augmented postprandial insulin rises prestimulated by a liver extract-sucrose test meal and intravenous glucose plus an amino acid mixture respectively. In rats, additions of β-casomorphin-4-amide or β-casomorphin-3-pyrrolidide, an analogue of β-casomorphin, in a diet containing wheat gluten also resulted in a stimulation of insulin (Brust *et al.*, 1991). Furthermore, a stimulation of insulin secretion by β-casomorphins was demonstrated in an *in vitro* study using isolated islets of Langerhans (Nieter *et al.*, 1981). A strong support for the insulinotropic actions of β-casomorphins is a widely accepted hypothesis that the μ-opioid receptor subtype is responsible for the stimulation of insulin secretion and the δ-opioid receptor subtype for the inhibition of insulin (Schusdziarra *et al.*, 1983a; Schick and Schusdziarra, 1985). Indeed, μ-agonists such as morphine (Kanter *et al.*, 1980) and DAMME (Pierluissi *et al.*, 1981) stimulated insulin release from pancreatic islet cell cultures, while leucine-enkephalin (Lord *et al.*, 1977; Kanter *et al.*, 1980), a δ-agonist, inhibited it. Thus the results in Experiment 1 and 3, considering that β-casomorphin is a relatively more potent agonist of the μ-opioid receptor type, may appear to be 'unexpected'.

However, there are also several reasons why the results of Experiment 1 and 3 may not be surprising. Firstly, there is evidence against the hypothesis of μ-opioid agonism and δ-opioid antagonism of insulin (Green *et al.*, 1983; Rudman *et al.*, 1983; Sullivan *et al.*, 1986). For example, Sullivan *et al.* (1986) reported that, in humans, intravenous morphine

infusion had no effect on plasma insulin when glucose was given intravenously while plasma concentrations of insulin were significantly decreased by the morphine when glucose was given by either oral or intraduodenal administrations. They suggested that the inhibition of insulin by morphine might be due to the inhibitory action of morphine on gastric emptying and intestinal motility, thus delaying the entry of the oral or intraduodenal glucose to the circulation.

Secondly, the insulinotrophic action of β-casomorphins, reported in the study of Schusdziarra *et al.* (1983a), does not obey the μ-opioid agonism rule since β-casomorphin-4-amide, the most potent and highly specific μ-agonist, did not stimulate insulin release whereas the infusion of β-casomorphin-4, an opioid peptide with about 40 times less potency than morphine, resulted in the greatest potentiation of insulin release (290 % higher than that of morphine), and the virtually opioid inactive β-casomorphin-3 was the second highest stimulant among the β-casomorphins used in the study. These results indicate that the action of β-casomorphins on insulin release reported in that study may not be mediated via binding with traditional μ-opioid receptors or, at least, there may be other system(s) or receptor type(s) involved in the process.

Thirdly, both the concentration of opioids applied and the dose-level of glucose in the experiments contribute significantly to the responses of insulin to the opioids. It is well-recognised that different concentrations of opioids can produce completely different metabolic and physiological responses including effects on plasma insulin and glucose concentrations (Green *et al.*, 1980; Nieter *et al.*, 1981; Ipp *et al.*, 1982; Johansen *et al.*, 1994). Indeed, this dose-dependent opioid effect applies to the actions of β-casomorphins on insulin secretion (Schusdziarra *et al.*, 1983a). Furthermore, the background concentration of glucose in plasma (*in vivo*) or in the incubation medium (*in vitro*) also seems to be an important factor affecting insulin responses to opioids since some studies reported lack of effects or even completely opposite effects of opioids (including β-

casomorphin) on insulin release in the presence of different glucose concentrations (Nieter *et al.*, 1981; Pierluissi *et al.*, 1981; Schusdziarra *et al.*, 1983a). A particularly interesting finding in this respect is that both β-casomorphin-4-amide and -5 stimulated insulin release by isolated islets of Langerhans of Wistar rats when the glucose concentration in the incubation medium was low (1.5 mmol / L glucose) whereas, at higher glucose concentrations (6 and 15 mmol / L glucose), insulin release was significantly inhibited by both peptides (Zuhlke *et al.*, 1994). In the same study, various concentrations (ranging between 10^{-4} and 10^{-8} M) of both β-casomorphin-3-Npyr and -4 significantly stimulated insulin biosynthesis in the isolated islets of Langerhans at low glucose concentrations (1.5 and 6 mmol / L) while, at 15 mmol / L glucose, the biosynthesis was inhibited by the peptides. These results are in accordance with the results of Experiment 3 where the inhibitory action of the β-casomorphin mixture on insulin was only evident when glucose was infused at the higher rate (plasma glucose concentrations at approximately 5 - 5.5 mmol / L) and the β-casomorphins had no effect on insulin at the lower glucose infusion (plasma glucose at approximately 4 - 4.5 mmol / L).

Finally, in addition to the complications mentioned above, as discussed in Chapter 5, species differences could also be responsible for the apparently conflicting results of the effect of β-casomorphins on insulin secretion. There is only one report concerning the possible involvement of the opioid system in the control of insulin release in ruminant animals. Froetschel *et al.* (1997) observed a decrease in the postprandial rise in insulin concentration after an abomasal infusion of naltrexone, an opioid blocker, in steers receiving a diet high in ruminally undegradable protein (RUP) and they speculated that the presence of opioid peptides in blood meal (hemorphin) incorporated in the RUP supplement fed in the study may have been responsible for the rise in insulin that was inhibited by the naltrexone. However, although interesting, this result is probably not relevant to the results of Experiments 1 and 3, since the opioid peptide concerned in the

study is different from that of the present study. Besides, naltrexone can influence the actions of endogenous opioids as well as exogenous ones, so complicating the interpretation of the findings.

The mechanism by which opioids modulate insulin release is not clear although some possible mechanisms have been postulated. These include direct action on B cells in islets of Langerhans (Rudman *et al.*, 1983); direct or indirect mediation by somatostatin (Ipp *et al.*, 1978; Schusdziarra *et al.*, 1984); involvement of the central nervous acetylcholinergic system (Salazar and Zuhlke, 1990); glucagon-induced changes in plasma glucose concentration (Schusdziarra *et al.*, 1984); and secondary mediation through inhibition of gut motility (Sullivan *et al.*, 1986). Since the inhibitory action of exogenous opioids on gut motility is well known, the decrease in insulin response to the abomasal glucose by the β-casomorphin mixture in Experiment 1 might have been due to the slow absorption of the glucose secondary to the inhibitory effect on motility. However, this possibility can probably be ruled out because there was no difference in the glucose concentrations between the treatments with or without the β-casomorphins in Experiment 1 and, what is more, the inhibitory action of the β-casomorphins was still evident when glucose was administered intravenously in Experiment 3.

Among the mechanisms, hormonal or neuronal mediation by somatostatin has received most attention as a predominant underlying mechanism for the opioid effects on insulin and glucose, especially when opioids are administered orally or direct into the gut. Contrary to the mechanism of effects on insulin, a rule of 'μ-opioid antagonism and δ-opioid agonism' seems to apply to somatostatin control by opioids (Hermansen, 1983; McIntosh *et al.*, 1990). Although this hypothesis is still controversial because of some exceptions (see Schick and Schusdziarra, 1985), it does fit in the case of the only available evidence of action of β-casomorphin on somatostatin release. In an *in vivo* study using conscious dogs, Schusdziarra *et al.* (1983b) reported that orally administered β-

casomorphins augmented the postprandial rise of peripheral vein plasma SLI levels significantly and the effect was inhibited by the administration of naloxone. This observation is particularly relevant to the present study because the administration method, the amount of the β-casomorphins (12 mg per dog weighing 24 - 36 kg) and the composition of the mixture of β-casomorphins (β-casomorphin-4, -4-amide, -5 and -7) were very similar to those used in the present study. Therefore, since SS-28 is largely responsible for the postprandial SLI (Chiba and Yamada, 1994), the inhibitory action of the β-casomorphins on insulin release observed in Experiments 1 and 3 could well be mediated by the stimulation of SS-28 released by the proximal intestinal epithelial cells.

On the other hand, in Experiment 2b where lactating cows were used, there was no effect of the mixture of β-casomorphins on the insulin response while the co-administration of β-casomorphin-4-amide resulted in an increase in insulin response to the glucose during the period of initial rise. This suggests that there may be different mechanism(s) involved in the control of metabolic hormones by opioids in lactating animals (see previous chapter). The stimulatory effect of β-casomorphin-4-amide alone, but not the mixture, on insulin secretion may be an indication of different actions by the different β-casomorphins used in the study although it would be necessary to carry out more investigations before drawing any conclusion. It is noteworthy that information on glucagon responses to the β-casomorphins in Experiment 2 would have been useful since there was a clear indication of stimulatory action by the peptides on glucagon in Experiment 2 of Chapter 5 where lactating animals were used.

CHAPTER SEVEN

GENERAL DISCUSSION

The results of the experiments have been discussed in detail in their respective chapters but there remains a need for a more general, integrated discussion.

The starting point for this thesis was that positive milk production responses to abomasal infusions of casein could not be explained solely by traditional interpretations based on the supply and demand for amino acids for milk protein synthesis. The inability to account completely for the superiority of casein over soya protein is well represented by figure 7-1 where approximately 35 % of the difference in response to the two proteins, casein and SPI, remained unaccounted for even after additions of amino acids to the SPI to make it equivalent to casein in terms of total amino acid content, total essential amino acid content and balance of the essential amino acids.

In investigating actions of β-casomorphin in dairy cows, as one of the possible explanations of the superiority of casein, certain assumptions had to be made mainly because of the lack of information on their actions in ruminants. For example, selection of individual β-casomorphins was based on the production of the peptides during digestion *in vitro* (Brantl *et al.*, 1979; Teschemacher *et al.*, 1980; Chang, *et al.*, 1985). However, there are two potential problems in the selection of the peptides. Firstly, although post-ruminal protease activities of ruminants are believed to be similar to gastric and intestinal protease activities of simple-stomached animals (see Chapter 1), the profile of β-casomorphins produced during post-ruminal digestion in dairy cows may be different from that observed in simple-stomached animals. Secondly, errors are possible in extrapolating from the *in vitro* data because variations in the production of β-casomorphins were found depending on the source of casein, the incubation method and the species used. Therefore, it should be recognised that, *in vivo*, the variety of β-casomorphins released in dairy cows may not

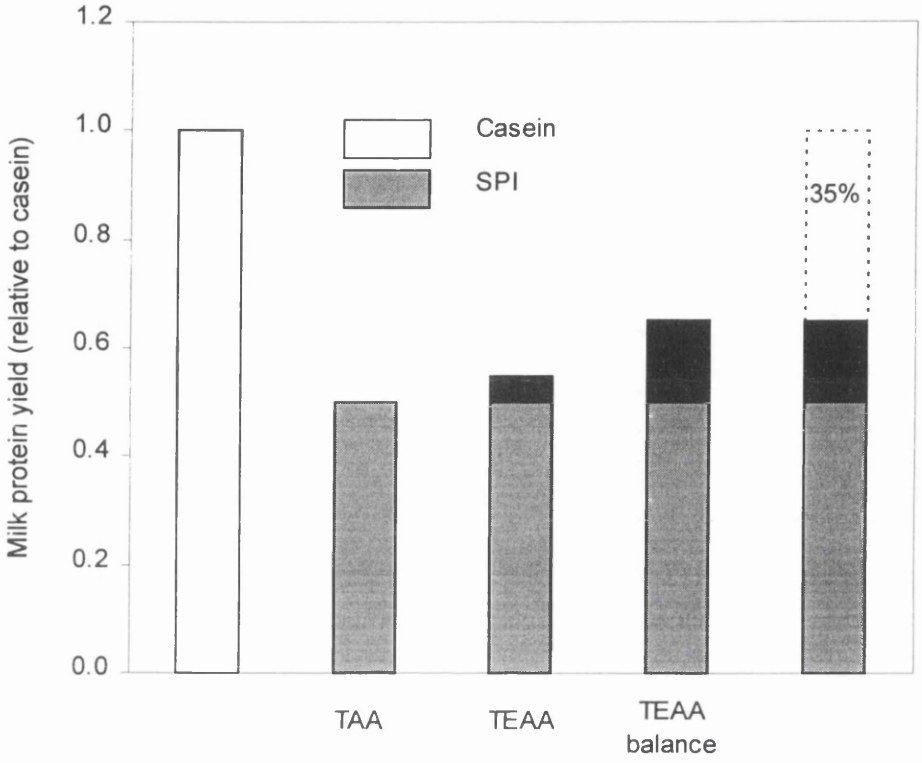


Figure 7-1. Effects of addition of amino acids to abomasal infusions of soya protein isolate (SPI) to make them equivalent to casein, in terms of total amino acid content (TAA), total essential amino acid content (TEAA) and balance of the total essential amino acids (TEAA balance), on the response of milk protein yield in dairy cows. Values are expressed relative to the response to infusion of casein which is 1.00 (Calculated from Choung and Chamberlain, 1992a).

accurately reflect those used in the experiments in this thesis. Furthermore, it has to be remembered that β-casomorphin-4-amide included in the mixture of β-casomorphins is unlikely to be natural, although it has been suggested that it might be produced naturally (Chang *et al.*, 1985). In addition to the difficulties associated with the uncertainty over the selection of the peptides, the dose levels of β-casomorphins used in the study could also be different *in vivo* because they were also calculated by extrapolating from observations made *in vitro* and *in vivo* in simple-stomached animals; the only data available *in vivo* reports 'β-casomorphin immuno reactive materials' rather than individual peptides.

The selection of the peptides and their dose levels becomes even more complex when the full spectrum of bioactive peptides from casein is considered. As shown in table 1-4 in Chapter 1, there are a number of peptides derived from casein that possess biological activities, some of them with opioid antagonism. Therefore, potential interactions of β-casomorphins with other bioactive peptides that might have opioid or non-opioid effects cannot be ruled out. However, although it was fully recognised that the selection of the peptides and their dose levels in the present study would, inevitably, be an oversimplification, a decision had to be made because of practical constraints. The type and doses of β-casomorphins selected were judged to be the best approximation to a representative mixture, as discussed in Chapter 6.

The investigations in this thesis were mainly focused on the effects of abomasal infusions of β-casomorphins on hormonal changes over a short period of time (3 to 7 hours) after the infusions. Although it was also recognised from the beginning of the study that β-casomorphins may have effects on gut motility (Kil and Froeschel, 1994), this issue was not dealt with in this thesis. In passing, it is worth noting that abomasal infusion of casein is often associated with increases of food intake and it is difficult to reconcile such effects with the reported increase of gut transit time by β-casomorphins in simple-stomached animals. However, there is a more practical reason why the investigations had

to be limited to short term observations rather than longer-term effects (e.g. milk yield, milk composition). The β-casomorphins used in the *in vivo* experiments of Chapters 5 and 6 alone amount to eight grams. To synthesise this quantity of peptides takes more than a month of exclusive use of a peptide synthesiser. For milk production trials, the total amount of the peptides required would be at least 10 times greater.

The results of experiments in Chapter 6 showed obvious differences between lactating and non-lactating cows in their responses of insulin to the abomasal infusion of β-casomorphins. Meeting the nutrient requirements of lactation necessitates various metabolic adaptations including increase in feed intake (Service *et al.*, 1983) and blood flow (Miller *et al.*, 1991), changes in concentrations of circulating metabolic hormones (Vernon *et al.*, 1981), sensitivity of hormonal response to substrates (Vernon *et al.*, 1990; Faulkner and Martin, 1999) and tissue responsiveness to hormones (Vernon *et al.*, 1981; Vernon and Taylor, 1988). Among these changes, decreased sensitivity of insulin to exogenous glucose in lactating animals is now well established (Faulkner and Martin, 1999) and this decrease appears to be evident in the observations made in the experiments of Chapter 6 where the average peak insulin response to the 100 g of abomasal glucose in non-lactating dairy cows was 2.86 ng / ml but, in lactating cows, it was only 2.23 ng / ml to the 200 g of abomasal glucose. It is tempting to suggest that the lack of insulin response to the β-casomorphins in lactating animals is due to the decreased sensitivity of insulin to its triggers. This might reflect also a reduced sensitivity of insulin to β-casomorphin's action in lactating cows which might have made the dose level (240 mg) of the administrated β-casomorphins in Experiment 2b of Chapter 6 too low. In any case, the failure to clearly demonstrate effects of β-casomorphins on insulin in lactating cows together with the short-term nature of the experiments makes it difficult to directly relate the observations in the present study to results of production experiments, and consequently, the present results do not provide an adequate test of the original hypothesis.

Nevertheless, in spite of these possible difficulties in the interpretation of the results, the reproducible insulinopenic action of the abomasal β-casomorphins in the non-lactating dairy cows, as demonstrated by Experiments 1 and 3 in Chapter 6, implies that casein-derived bioactive peptides can influence hormone levels in dairy cows. Such effects, if they occur in lactating cows, might be expected to result in a repartitioning of nutrient use between non-mammary and mammary tissues, which is often observed following post-ruminal casein infusions (see Chapter 1). Moreover, the directions of the observed changes in concentrations of various hormones (e.g. inhibition of insulin and stimulation of glucagon and GH) following the abomasal infusion of β-casomorphins are generally in line with the hormonal changes that would be expected to benefit milk synthesis by the mammary gland (see Chapter 1). This warrants more detailed investigation of their actions in controlling metabolic hormones, especially GH and glucagon. In addition, because the ruminant absorbs only small amounts of glucose from the small intestine (Merchen, 1988) and propionate is the principal glucogenic nutrient absorbed by ruminant animals (Thomas and Rook, 1983), it would be interesting to see whether the inhibitory action of β-casomorphins occurs when the insulin is augmented by pretreatment with propionate or even amino acids.

Although a likely mechanism underlying the insulinopenic effect of the abomasal infusion of the β-casomorphins seems to be mediation by SS-28, presumably via interactions with the SS-28 secretory cells in the small intestine as discussed in Chapter 5, the site of the action of β-casomorphins is still not clear. Indeed, even their ability to pass through the intestinal barrier remains debatable. In view of this, demonstration of hormonal responses to intravenously administered β-casomorphins might provide further information on the underlying mechanism. Alternatively, *in vitro* approaches using isolated bovine islets of Langerhans and various preparations of intestinal tissues could enable more intensive investigations on the mechanism of the action of β-casomorphins.

It should be recognised that, at this stage, any implications that can be drawn from the results of the present study must be speculative. If the insulinopenic action of β-casomorphins in non-lactating dairy cows were to be confirmed in lactating dairy cows, this would highlight a potential use of the peptides as possible production enhancers in dairy cows, although further extensive experimentation would be needed to identify the nutritional and physiological circumstances in which their use would be beneficial. For this purpose, development of easy and effective methods of administering the peptides would be an essential step. An example of such a method could be the preparation of 'capped N-acetyl β-casomorphins'. Although N-terminal acetylation could be used as a simple and effective method to protect the peptides from the hydrolysis in the rumen, their release in active form in the small intestine cannot be assured. It would, therefore, be necessary to introduce a 'capped design' in combination with the acetylation so that the active β-casomorphins can be delivered by simply adding the modified peptides in the diet of ruminant animals (figure 7-2). If this approach were successful, an added advantage would be that *in vivo* investigations of the actions of β-casomorphins or similar bioactive peptides in ruminant animals would become less demanding in terms of experimental technique because surgically modified animals would not be needed.

Furthermore, this study suggests that there may be an additional feature of the digestion and metabolism of feed proteins to be considered in nutritional evaluations. β-Casomorphins may not be of importance in practice for dairy cows in this sense since casein is not a typical component of diets for dairy cows. However, release of peptides with a similar biological activity to β-casomorphins is believed to occur during digestion of other protein sources such as wheat gluten and haemoglobin (Fukudome *et al.*, 1997; Nyberg, 1997) which could be of more immediate relevance in practice. Furthermore, the

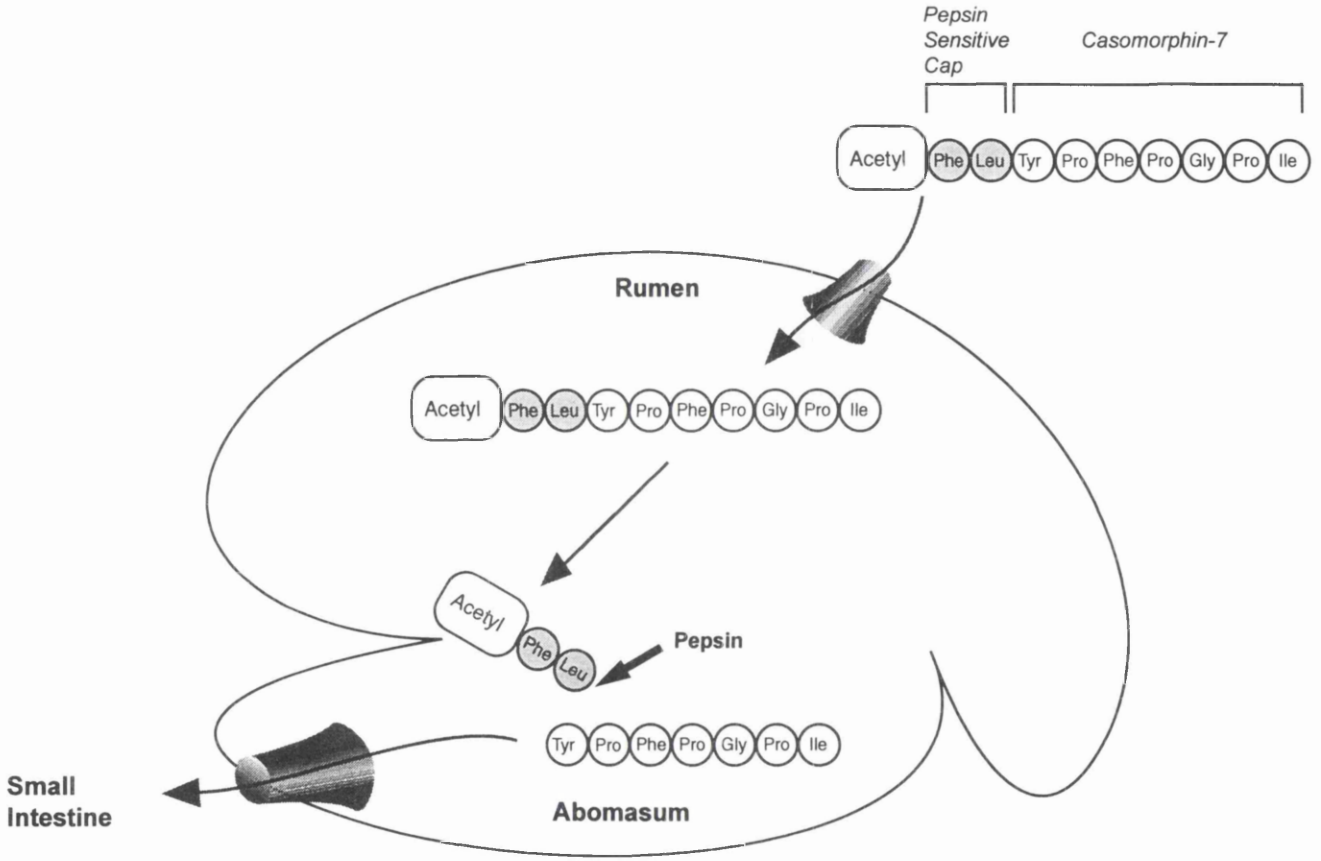


Figure 7-2. The 'acetyl-capped-casomorphin' concept.

list of such peptides is likely to continue to grow as the structure of more proteins comes under scrutiny.

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