EFFECTS OF AMINO ACID NUTRITION ON MILK PRODUCTION RESPONSES OF THE DAIRY COW TO MORE FREQUENT MILKING

JOON-MO YEO

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Hannah Research Institute, Ayr, Scotland

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SUMMARY

1. This thesis focuses on the influence of amino acid nutrition on the response of lactating dairy cows to increasing the frequency of milking from twice to thrice daily. Two diets were chosen to represent extremes of amino acid balance. A basal diet of grass silage was supplemented with a cereal-based concentrate containing either fish meal or feather meal. Fish meal and feather meal have a similar, high content of rumen-undegradable protein (UDP) but differ widely in amino acid composition. Compared with fish meal, feather meal contains low concentrations of histidine, methionine and lysine. Both diets were given in amounts calculated to supply metabolizable energy (ME) well in excess of requirement (at least 15 %).

2. As a starting point, two experiments were conducted to examine the pattern of response of milk production and mammary function (total DNA, cell proliferation rate, activities of key enzymes) to differences in amino acid supply in two stages of lactation. A control group received the fish meal diet throughout the experiments whereas cows in the treatment group received the fish meal diet until week 6 of lactation when they were changed to the feather meal diet for 6 weeks and then returned to the fish meal diet for 4 weeks (Experiment 1). After a break of 5 weeks, the experimental procedure was repeated using the same cows (Experiment 2). Although milk yield fell as lactation advanced, the differences in milk yield between the feather meal and the fish meal treatments in the two stages of lactation remained similar (21 % and 16 % in Experiments 1 and 2, respectively). However, despite the markedly lower milk yield in cows given the feather meal diet, no clear differences between the treatments were detected in the measurements of mammary function.

3. A series of three experiments was conducted to test the hypothesis that cows are unable to fuel an increase of milk production in response to an increase of milking frequency when milk production is limited by deficiencies in the supply of specific amino acids (His, Met and Lys). The first two experiments were conducted in late (Experiment 3) and early (Experiment 4) lactation to examine the effect of stage of lactation on the response to thrice-daily milking in cows consuming a diet containing feather meal as the only protein supplement. In addition, in Experiment 4, half-udder milking was adopted to examine compensatory responses in milk yield between the two halves of the udder in response to thrice-daily milking. In the third experiment (Experiment 5), fish meal, a positive control, was included against feather meal and the response of thrice-daily milking to an increase of ME supply was also investigated.

The results of Experiment 3 and 4 show that, in cows fed a diet containing feather meal as the only protein supplement, thrice-daily milking had little or no effect on milk secretion in the two stages of lactation. Although no positive dietary control was included in Experiments 3 and 4 owing to the lack of available cows at the time, the results of Experiment 5 confirmed that, with the feather meal diet, milk yield was not affected by thrice-daily milking. However, with the fish meal diet, milk yield increased in response to thrice-daily milking (25.0 and 26.6 kg/d for 2x and 3x milking, respectively; $P<0.05$). Additional ME supply on the fish meal diet did not increase milk yield beyond the level of that with the fish meal diet for either twice- or thrice-daily milking. When compensatory responses were examined (Experiment 4), it would seem that a small, but significant decrease in milk yield from the glands milked twice daily contributed to the apparent higher milk yield in the glands milked thrice daily. Increasing the frequency of milking did not affect the ratio of 3-methylhistidine/creatinine in urine, suggesting that the rate of degradation of muscle protein was unchanged.

4. Experiment 6 investigated whether growth hormone, a potent re-partitioning agent, might provide a stronger stimulus to repartitioning of amino acid use and so allow an increase of milk yield in response to an increase of milking frequency even when the basal diet was deficient in amino acids. The four treatments were feather meal with (FE + bST) or without bST (FE) and fish meal with (FI + bST) or without bST (FI). One half-udder was milked thrice daily (3x) and the other half milked twice-daily (2x). Milk yields were 19.0, 22.0, 24.4 and 27.3 kg/d (SED 0.81) for FE, FE + bST, FI and FI + bST treatments, respectively. As before, an increase in the secretion of milk protein in response to thrice-daily milking was blocked on the feather meal diet. However, the yield of milk protein with this diet was markedly increased by around 10 % when the cows were injected with growth hormone. In contrast, with the fish meal diet, responses of milk production to growth hormone and to milking more frequently were additive.

5. Overall, the results of the experiments with cows given the feather meal diet show that, even though dietary ME is in considerable excess, a deficiency of specific amino acids (His, Met and Lys) can prevent any increase in milk yield in response to increasing the frequency of milking from twice to thrice daily. In contrast, when cows consumed a similar level of excess ME and a similar level of rumen-undegradable protein where the protein was of better amino acid balance (fish meal), the increased frequency of milking led to increased milk yield. The conclusion is that (a) the amino acid nutrition of the dairy cow modulates the response to an increase in milking frequency, and (b) compared with growth hormone injection, increasing the frequency of milking has only a weak effect on the partitioning of amino acid use between body and udder.

DECLARATION

All animal experimentation was carried out by me. With the exception of feed, milk, urine and AA analysis by Mrs I. Stewart and Mr J. Davidson, I declare that the work contained in this thesis is my own, undertaken under the supervision and guidance of Drs D. G. Chamberlain and C. H. Knight. The work in this thesis has not been submitted for any other degree or qualification.

Joon-Mo Yeo

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LIST OF ABBREVIATIONS

2x	Twice-daily milking
3x	Thrice-daily milking
AA	Amino acid
ACC	Acetyl CoA carboxylase
ADF	Acid detergent fibre
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bST	Bovine somatotropin
DM	Dry matter
DNA	Deoxyribonucleic acid
EAA	Essential amino acid
EDTA	Ethylene diaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FAS	Fatty acid synthetase
FIC	Fish meal cube
FMC	Feather meal cube
GH	Growth hormone
GT	Galactosyltransferase
HPLC	High-performance liquid chromatography
IGF-I	Insulin-like growth factor-I
IgG	Immunoglobulin G
ISTD	Internal standard

MBF	Mammary blood flow
ME	Metabolizable energy
MP	Metabolizable protein
NDF	Neutral detergent fibre
NEAA	Nonessential amino acid
NEFA	Nonesterified fatty acids
PCNA	Proliferating cell nuclear antigen
PEG	Polyethylene glycol
RDP	Rumen-degradable protein
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SED	Standard error of differences
SEM	Standard error of means
UDP	Rumen-undegradable protein
VFA	Volatile fatty acids
WSC	Water-soluble carbohydrate

CHAPTER ONE

REVIEW OF LITERATURE

1.1 INTRODUCTION

Chapter one of this thesis reviews the literature relating to effects of frequency of milking on the secretion of milk, with particular reference to interactions with nutrient supply.

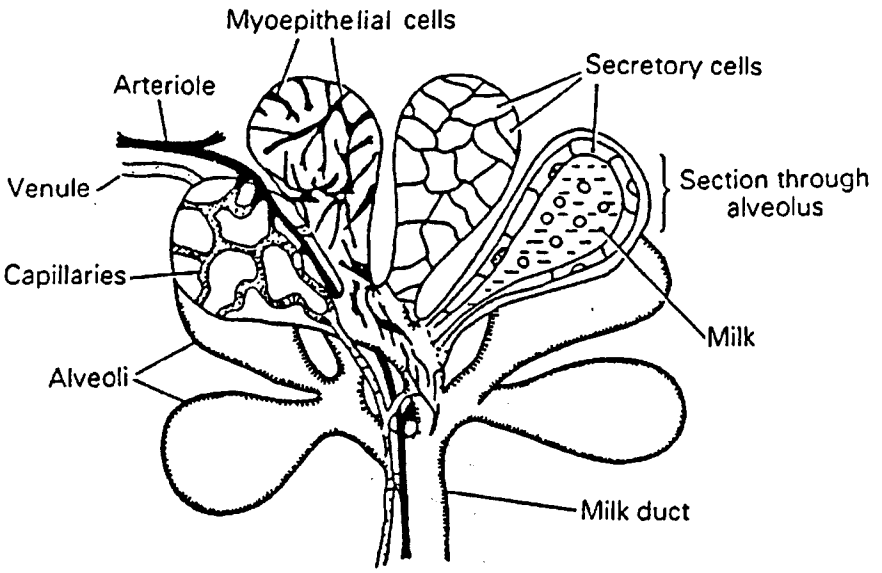
1.2 BOVINE MAMMARY GLAND

1.2.1 The structure of mammary gland

The bovine mammary gland consists of an udder which is composed of four inguinal glands. The fully developed mammary gland is composed of two main components, parenchyma and stroma. The parenchyma of mammary gland consists of epithelial cells (secretory cells), myoepithelial cells and ductal tissue. Those elements of the mammary tissue not included in the parenchyma are collectively called the stroma which includes the skin, connective tissue, adipose tissue, blood and lymph vessels and nerve tissue.

The epithelial cells are grouped in numerous pear-shaped alveoli, in which the cells are arranged in a single layer lining the hollow lumen (Figure 1.1). Groups of neighbouring alveoli, drained via a common duct, constitute a lobule. Some cells of the smaller ductules may also be capable of milk secretion and these, together with the alveoli, are collectively termed the lobuloalveolar system. Milk is secreted into the hollow of the alveoli by contraction of myoepithelial cells, from which it drains via narrow ductules into larger-bore ducts which constitute the duct system, and accumulates in the cisterns (Figure 1.1).

(a)



(b)

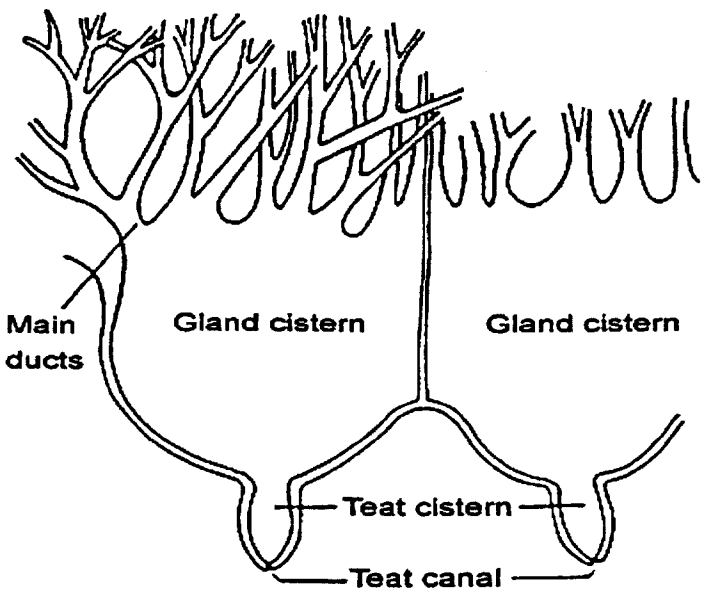


Figure 1.1 The structure of bovine mammary gland (Mepham, 1987). (a) Diagrammatic representation of a cluster of mammary alveoli. (b) Arrangement of mammary duct system.

1.2.2 The development of mammary gland

At birth, the mammary gland consists of a restricted immature duct system and a stromal portion. Before puberty the ducts elongate into the stromal portion of the mammary gland with an accompanying increase in stroma (Tucker, 1987). The mammary ducts begin to grow at a faster rate than general body growth (allometric growth) well in advance of the first oestrous cycle. Allometric mammary growth continues for several oestrous cycles, then returns to an isometric pattern (mammary growth rate equals general body growth) until conception (Sinha and Tucker, 1969). After conception, growth of mammary parenchyma increases exponentially throughout gestation. In early pregnancy, the growing mammary gland consists primarily of densely packed tubules. In mid pregnancy, small alveoli containing secretory material are formed, and increase in number and size until parturition (Swanson and Poffenbarger, 1979).

Total DNA content of bovine mammary gland, indicating mammary cell numbers, continues to increase during the early stage of lactation (Akers *et al.*, 1981). After peak lactation, the mammary cell numbers decline with advancing lactation (Capuco *et al.*, 2001). Following cessation of milking, the tissue area occupied by alveolar or ductal lumina decreases with a concurrent increase in the stromal area (Holst *et al.*, 1987; Capuco *et al.*, 1997). Unlike that of rodents, the bovine mammary gland does not show extensive sloughing of epithelial cells from the basement membrane into the alveolar lumen during the dry period, suggesting that there is little net loss of cells during the dry period (Holst *et al.*, 1987; Hurley, 1989). Furthermore, mammary cell numbers do not differ between dry and lactating cows in their final week of gestation (Swanson *et al.*, 1967; Capuco *et al.*, 1997). However, increased apoptosis and cell proliferation in the mammary gland during the dry period, relative to that in lactating glands during the same

stage of gestation, suggest that a dry period serves to promote cell turnover prior to the next lactation (Capuco *et al.*, 1997; Capuco and Akers, 1999).

1.2.3 Control of mammary development

It is well recognized that oestrogen stimulates mammary duct growth, and a combination of oestrogen and progesterone synergically stimulates lobuloalveolar development (Lyons, 1958; Cowie and Folley, 1961).

In addition to ovarian steroids, maximal mammary growth requires growth hormone, prolactin and placental lactogen (Lyons, 1958). It has been shown that the detrimental effect of high energy intake on mammary development in prepubertal and peripubertal heifers is associated with decreased concentration of growth hormone in blood serum (Sejrsen *et al.*, 1983). Indeed, administration of growth hormone to growing heifers increases mammary development (Sejrsen *et al.*, 1986). Growth hormone may exert its effect indirectly on mammary growth through the effect of insulin-like growth factor-I (IGF-I) because there is a lack of growth hormone binding to mammary gland in the dairy cow (Sejrsen *et al.*, 1999). However, a direct effect of growth hormone cannot be excluded since growth hormone receptor protein and mRNA have been found in the bovine mammary gland (Sinowatz *et al.*, 2000; Plath-Gabler *et al.*, 2001).

In dairy cows, prolactin concentrations in blood show seasonal variations, being positively correlated with daylength and environmental temperature. Increase of prolactin concentration in blood in response to extending photoperiod stimulates mammary growth in prepubertal and postpubertal heifers (Petitclerc *et al.*, 1985). Prolactin, with withdrawal of progesterone at parturition, is also involved in the initiation of lactation, being markedly elevated during the last few days before parturition in the dairy cow (Mepham,

1987; Knight, 2001). However, it is very unlikely that prolactin is involved in the control of milk production in the dairy cow (Karg *et al.*, 1972; Knight, 2001).

As the structure of placental lactogen is related to that of prolactin, a role for prolactin in mammary growth might be expected. Although the concentration of placental lactogen is very low in maternal serum relative to that in the fetus, it is capable of increasing the mammogenic action of exogenous steroids in heifers (Byatt *et al.*, 1994).

Little is known about hormonal control of mammary cell number during lactation in the dairy cow. However, growth hormone treatment can maintain mammary cell number in lactating goats (Knight *et al.*, 1990). Recently, Capuco *et al.* (2001) also showed that administration of growth hormone to lactating cows reduced the rate of mammary regression and increased cell proliferation. Besides hormones, local factors may also be involved in the control of mammary development during lactation because the mammary gland is known to produce both growth factors and inhibitors of cell proliferation (Wilde and Knight, 1989; Knight and Wilde, 1993). It has been suggested that, in lactating goats, increased mammary cell number in response to an increase of milking frequency resulted from more frequent removal of a chemical inhibitor (FIL, feedback inhibitor of lactation) present in milk (Wilde *et al.*, 1987a). There are indications that the chemical inhibitor present in milk is in the whey protein fraction (Wilde *et al.*, 1987a), but its identity has not yet been established.

1.3 MILK SYNTHESIS AND SECRETION

Lactose is the main osmotic constituent in milk and its rate of secretion regulates milk yield. Glucose is the primary substrate for lactose synthesis. In addition, glycerol derived from plasma triglycerides, lactate and non-essential amino acids contribute to the glucose pool within the mammary gland. Glucose is partly converted to UDP-galactose in

the cell cytosol, and glucose and UDP-galactose combine together in the Golgi apparatus to form lactose under the action of the enzyme lactose synthetase, which consists of galactosyl transferase and α-lactalbumin (Kuhn *et al.*, 1980).

Milk protein consists of a mixture of α_{s1}-, α_{s2}-, β-, κ- and γ-caseins, α-lactalbumin, β-lactoglobulin, proteose peptones, immunoglobulin and bovine serum albumin. The main components and the major proportion of milk protein are synthesized in the mammary gland, and a little urea enters the milk by diffusion from plasma (Thomas, 1983). The processes of milk protein synthesis are similar to those which apply to other cellular proteins, amino acids being assembled under the direction of mRNA, tRNA and rRNA at the endoplasmic reticulum of the cell. As the milk proteins are synthesized they are transported to the Golgi apparatus for packaging prior to secretion. The proteins and lactose formed within the Golgi region are encapsulated together with water and ions, and form vesicles which bud from the apical surface of the Golgi apparatus and move to the apical surface of the cell, from where they fuse with the membrane and disgorge their contents into the alveolar lumen (Mepham, 1987).

Most of cow's milk fat (98 %) is triglyceride, the major fatty acids of which contain 4 – 18 carbons and are even numbered. The short-chain ($C_4 - C_{10}$) and medium-chain ($C_{12} - C_{16}$) fatty acids are synthesized in the mammary gland from acetate and 3-hydroxybutyrate. However, a proportion of the medium-chain acids is taken up mainly from preformed lipoprotein triglycerides. The long-chain (C_{18}) fatty acids are derived entirely from blood plasma sources. The main pathway for fatty acid synthesis in the gland is the malonyl pathway, which involves malonyl-CoA formation from acetyl-CoA by acetyl-CoA carboxylase, and then fatty acid formation by fatty acid synthetase (Moore and Christie, 1981). As triglyceride is synthesized, by esterification of fatty acids with glycerol-3-phosphate, it forms droplets. The droplets increase in size, move towards the

apical membrane of the cell and are secreted by pinocytosis, emerging as fat globules bounded by a unit membrane derived from the cell membrane (Mepham, 1987).

1.4 CONTROL OF MILK SECRETION

Various factors are involved in control of milk secretion such as the arterial concentration of substrates, blood flow, the efficiency of extraction of substrates by the gland, and the number and the synthetic activity of secretory cells in the mammary gland, which may potentially be influenced by systemic (e.g. nutrition, hormones) or local (chemical or physical inhibition) factors. The relative importance of the factors may also change during the period of lactation.

1.4.1 Systemic factors

1.4.1.1 Nutrition

The main products of digestion absorbed from the rumen, small intestine, caecum and colon are volatile fatty acids (principally acetate, propionate, butyrate), glucose, amino acids and medium- and long-chain fatty acids. The composition of the mixture of digestion end-products alters in response to changes in the chemical composition of the diet. However, owing to the major effect of the microbial fermentation processes in the rumen, the quantitative relationships between the diet and the products of digestion are complex. Thus the effects of a change in diet on milk secretion generally reflect the cow's response to a complex series of alterations in nutrient supply (Thomas and Martin, 1988).

Carbohydrate-rich feeds are the main sources of energy-yielding constituents in the cow's diet. The carbohydrate composition of the diet has a major effect on the molar proportions of acetate, propionate and butyrate in the rumen. However, the amount and

type of carbohydrate also influence the intestinal uptake of glucose and, via effects on the synthesis of microbial protein in the rumen, amino acids (Sutton, 1985).

Increases in energy intake are most commonly achieved through the additional allowance of supplementary foods, generally starchy concentrates. Relationships between milk yield, liveweight gain and metabolizable energy (ME) intake are well described by curvilinear functions (Blaxter, 1962; Broster, 1976), the responses in milk yield diminishing and the responses in liveweight gain increasing with each successive increment in level of feeding. These curves reflect a continuous competition for nutrients between milk secretion and the other synthetic processes in the body. Increase in ME intake associated with increases in starchy concentrate intake leads to some reduction in forage intake in cows given forage *ad libitum* and an increase in the dietary content of readily fermentable carbohydrate. These modifications in diet have effects on milk composition, including reductions in milk fat content and, less reliably, increases in protein content (Thomas, 1983). Reductions in milk fat content in cows receiving diets having a low ratio of forage to concentrate are probably due to a reduced ruminal production of acetate and butyrate, since these acids are the main precursors for short- and medium-chain fatty acids in the mammary gland (Thomas and Chamberlain, 1984). However, the effects of high-concentrate diets on milk fat synthesis may also be hormonally mediated, the elevated plasma insulin changing nutrient partitioning towards adipose tissue (Sutton, 1984). The increase in milk protein content in response to additional allowance of starchy concentrate may be related to the effect of increase of propionic acid supply by 'sparing' amino acids for gluconeogenesis in the liver (Rook, 1976). Another suggestion is that milk-fat depression is caused by a derangement of lipid metabolism in the rumen characterized by increased escape of *trans* isomers of unsaturated fatty acids (Davis and Brown, 1970). This theory has gained support from the

recently reported observation that conjugated linoleic acid markedly depresses milk fat content (Bauman and Griinari, 2001).

Changes in the amount and type of dietary protein have both direct and indirect effects on the amino acid supply to the cow's small intestine. The former depends on the amount and amino acid composition of the rumen-undegradable protein fraction (UDP), while the latter reflects the influences of diet composition on microbial protein synthesis in the rumen (Thomas and Martin, 1988). Microbial protein synthesis depends largely on the supply of energy (ATP) which is derived from the rumen fermentation of dietary digestible organic matter and the supply of ammonia which is released from the breakdown of dietary rumen- degradable protein (RDP) (Agricultural Research Council, 1984). There is also evidence that microbial protein synthesis in the rumen may be enhanced through bacterial incorporation of amino acids or peptides (Maeng *et al.*, 1976).

Milk production responds to changes in both the amount of dietary protein and its amino acid composition (Rulquin and Verite, 1993). However, these findings can be difficult to interpret because the amino acid supply to the post-ruminal gut cannot be accurately predicted owing to technical difficulties in the measurement of the degradability of dietary proteins and the synthesis of microbial protein in the rumen (Oldham, 1994). It is also necessary to note that feed intake is often increased in response to changes of protein level in the diet through improving digestibility, which increases the amounts of amino acids and other nutrients available to the animal from the basal diet (Oldham, 1984).

To overcome the difficulty of interpreting milk production responses to protein supplements, some workers have used the direct infusion of protein, usually casein, into the abomasum. It has been shown that casein infusions increased milk production in cows receiving basal diets varying in crude protein content between 11 % and 24 % (Thomas

and Chamberlain, 1984). Furthermore, in some studies, casein infusion increased the output of energy in milk by amounts far greater than the energy supplied by the casein itself, suggesting that the effect of casein on milk production is partly due to a repartitioning of nutrient use between body tissue and milk synthesis (Choung and Chamberlain, 1993) or a stimulation of body tissue mobilization (Ørskov *et al.*, 1977). The regulation of such a response might be endocrine in nature, involving especially growth hormone and insulin (Oldham, 1994).

Clear responses of milk production to different amino acid compositions in protein supplements were seen in experiments where comparisons were made between isonitrogenous infusions of casein and soya protein isolate (SPI) into the abomasum (Choung and Chamberlain, 1992, 1993). The response of milk production to casein infusion was much higher than to SPI, suggesting that the amino acid composition of casein is superior to that of SPI. Consistent with these effects of amino acid composition of dietary protein, are the well-documented responses of milk secretion to supplements of specific limiting amino acids such as methionine and lysine (Schwab *et al.*, 1992; Rulquin and Verite, 1993).

1.4.1.2 Hormones

Even when the supply of nutrients is adequate, there are marked differences in the extent to which lactating cows partition nutrients preferentially towards either milk or body tissues, and these differences are apparent both between animals of different yield potential and between different stages of lactation within animals. These variations are genetic in origin and probably are mediated via differences in hormonal balance (Bines and Hart, 1982).

In early lactation, the concentration of growth hormone in plasma is high in high-yielding cows relative to that in low-yielding cows, and declines as lactation advances (Hart *et al.*, 1978; Sartin *et al.*, 1988), indicating that growth hormone secretion is positively correlated with milk yield (Hart *et al.*, 1979; Hart, 1983). Moreover, it is well known that administration of recombinant bST (bovine somatotrophin), exogenous growth hormone, can change nutrient partitioning and increase milk production substantially in dairy cows (Johnsson and Hart, 1986; Bauman, 1992; Bauman and Vernon, 1993; Burton *et al.*, 1994; Etherton and Bauman,1998). Increase of food intake in response to bST occurs after a few weeks of bST treatment, but digestibility of the diet, the energy expenditure for maintenance and the partial efficiency of milk synthesis are not altered in response to bST (Bauman, 1992; Bauman and Vernon, 1993). Thus, until the point at which food intake is increased, nutrients required for increased milk production with bST treatment are supplied by mobilizing body reserves or changing nutrient partitioning.

The substantial response of milk production to bST requires orchestration of diverse physiological processes in a number of tissues and involves the metabolism of all nutrient classes. These adaptations involve both direct effects on the adipocyte and the hepatocyte, and indirect effects, via the IGF-I system, on the mammary gland (Bauman and Vernon, 1993). It has been reported that bST increased hepatic gluconeogenesis and lipolysis in adipocytes, and decreased lipogenesis (Bauman *et al.*, 1988; Cohick *et al.*, 1989; Bauman and Vernon, 1993). It is pertinent to note that a large part of the re-partitioning effect of growth hormone can be achieved via changes in cardiac output and rate of blood flow to the mammary gland (Mepham *et al.*, 1984; Davis and Collier, 1985; Fullerton *et al.*, 1989).

Increase of milk production in response to bST is observed in cows of all parities, but the magnitude of the increase in milk production varies according to stage of lactation, the response being small or negligible in early lactation prior to peak yield (Bauman, 1992; Bauman and Vernon, 1993). It has been suggested that, when cows are in negative energy balance during early lactation, a lack of response of milk yield to bST is probably related to little change in IGF-I concentration in blood which is normally increased by bST treatment in positive energy balance (Vicini *et al.*, 1991). Furthermore, little response in IGF-I concentration and milk production to bST in cows starved for two days in mid lactation (McGuire *et al.*, 1995) suggests that variations in IGF-I response to nutritional status may explain part of the variation in response of milk yield to bST. Inconsistent responses of milk yield to IGF-I infusion in lactating goats, associated with its binding proteins, add more complexity to the role of IGF-I in mediating the effect of bST on milk production (Davis *et al.*, 1989; Prosser and Davis, 1992).

In contrast to the catabolic action of growth hormone referred to above, insulin is involved in many anabolic actions in ruminants, as in non-ruminants. Glucose uptake and utilization by peripheral tissues are stimulated by insulin, whereas gluconeogenesis and glucose release from liver are inhibited. Insulin also stimulates synthesis of protein in muscle and lipid in adipose tissue, and inhibits proteolysis and lipolysis (Bassett, 1975; Vernon, 1988).

That high-yielding cows have low concentrations of insulin in plasma relative to low-yielding cows (Hart *et al.*, 1978; Sartin *et al.*, 1988) and that insulin administration decreases milk production (Kronfeld, *et al.*, 1963; Schmidt, 1966) suggest that the hormone affects nutrient partitioning such that nutrients are diverted away from milk synthesis and towards body tissues. These findings are consistent with plasma insulin

being negatively correlated with milk yield (Koprowski and Tucker, 1973; Hart *et al.*, 1979).

Although it has been proposed that insulin is responsible for 'low milk-fat syndrome' in cows receiving a high dietary ratio of concentrate to forage (Annison, 1976, Sutton, 1984), the underlying mechanisms of milk fat depression are still not clear. As mentioned earlier (see above), increases of circulating insulin in plasma, linked with high molar proportions of propionic acid in the rumen from feeding a high-concentrate diet, may lead to a diversion of lipogenic precursors towards adipose tissue at the expense of mammary gland, leading to a depression of milk fat synthesis (Sutton, 1984). This is further supported by the finding that more frequent feeding of cows eating high-concentrate diets reduces the mean daily concentration of insulin and leads to a corresponding lessening of the depression in milk-fat (Sutton, 1988). However, these results are not consistent with results from experiments in which insulin was infused and milk fat content was not affected (Schmidt, 1966; McGuire *et al.*, 1995a; Griinari *et al.*, 1997). Besides the effect of insulin on milk fat synthesis, the hyperinsulinaemic-euglycaemic clamp has been reported to increase both milk protein content and yield (Griinari *et al.*, 1997a; Mackle *et al.*, 1999), suggesting a potential stimulatory role of insulin on milk protein synthesis in dairy cows. But the results are difficult to interpret because the effects of insulin are compounded with the effects of an increased ME intake.

1.4.2 Local factors

1.4.2.1 Physical factors

As milk accumulates in the mammary gland, intramammary pressure begins to increase owing to the reduced volume available for storing the synthesized milk (Tucker *et al.*, 1961), and the rate of milk secretion starts to decline when intramammary pressure

is raised to a level that normally occurs following cessation of milking (Fleet and Peaker 1978; Peaker, 1980). The fact that the rate of milk secretion fell to zero within 1-2 days in goats during late lactation when the amount of milk accumulated after cessation of milking was replaced by isosmotic lactose solution and normal milking was continued indicates that the decline of milk secretion after cessation of milking is caused not by withdrawal of the milking stimulus or chemical inhibitors in milk, but by mammary distension itself resulting from increase of intramammary pressure (Fleet and Peaker, 1978). Furthermore, from results of short-term studies in which intramammary pressure was raised up to a physiological level with an isosmotic sucrose solution, it was proposed that the decreased milk secretion in response to the increase of intramammary pressure was probably due to a loss of secretory activity of the alveolar cell caused by altering the cytoarchitecture of the alveolar cells and to a secondary reduction in blood flow as a result of a decrease in the production of vasodilator substances (Peaker, 1980). Similarly, during 36 hours of milk accumulation in goats in mid lactation, decrease of blood flow and leaky tight junctions coincided with the start of the decline in milk secretion after 19 – 21 hours of milk accumulation (Stelwagen *et al.*, 1994a). Although the level of intramammary pressure was not measured in this experiment, it had been suggested that milk secretion, based on calculations of pressure-volume curves, would be expected to fall at 16 – 18 hours after the last milking in goats (Peaker, 1980).

The rate of milk secretion in cows begins to fall approximately 12 – 18 hours after the last milking (Turner, 1955; Elliot, 1959; Schmidt, 1960; Davis *et al.*, 1998) and therefore an extended milking interval, such as with once-daily milking, leads to a loss of milk production relative to twice-daily milking. The size of the cistern has been reported to be a possible limiting factor during once-daily milking; the larger the cisternal volume, the lower the production loss (Knight and Dewhurst, 1994; Stelwagen *et al.*, 1996; Davis

et al., 1998). However, it is uncertain to what extent mammary pressure exerts its effect on milk secretion during once-daily milking since milk is known to contain a chemical inhibitor (see below). It is unlikely, however, that the relief of mammary pressure is responsible for increases of milk secretion in thrice-daily milking. When one gland was infused with an amount of isosmotic sucrose solution, equal in volume to the milk removed at one of the three milkings in goats, the rate of milk secretion still increased relative to that of the gland milked twice daily, showing that a chemical inhibitor in milk, rather than physical distension, is responsible for the increase of milk secretion in thrice-daily milking (Henderson and Peaker, 1984).

1.4.2.2 Chemical factors

The suggestion that a chemical inhibitor in milk is involved in regulating milk secretion stems from the finding that milking transplanted glands, i.e. denervated glands, every hour in goats increased their milk yield relative to that of glands milked normally but manual stimulation without milk removal failed to increase milk yield (Linzell and Peaker, 1971). Evidence was obtained that it is the presence of milk in the secretory alveoli that is responsible for inhibition. When milk was drained from the duct system by catheter every hour in goats, a procedure that does not elicit the milk-ejection reflex, the rate of milk secretion did not increase but, with the administration of oxytocin to induce alveolar emptying, the rate of milk secretion increased (Henderson and Peaker, 1987). Furthermore, the frequent injection of oxytocin had no significant effect on secretory rate of the unmilked gland, showing that milk must be removed not only from the alveoli but also from the gland for milk secretion to increase. Studies on the effect of milk fractions on lactose and casein synthesis by rabbit mammary explants in organ culture revealed that a milk fraction containing whey proteins with a molecular mass of between 10 and 30

kDa, named feedback inhibitor of lactation (FIL), was involved in inhibiting milk secretion (Wilde *et al.*, 1987). Further, when FIL was infused into one mammary gland of lactating goats, it produced a temporary dose-dependent reduction in milk yield in the treated gland but the milk yield of the other gland, which received an equal volume of carrier solution, was not affected (Wilde *et al.*, 1988). Thus, the acute response of increased milk yield to frequent milking can be ascribed to the more frequent removal of FIL (Wilde and Knight, 1989; Knight and Wilde, 1993; Knight *et al.*, 1998). In addition, frequent milking of goats has been reported to increase, possibly via altered hormone sensitivity in the secretory cell, not only the activity of key enzymes regulating milk synthesis in mammary gland in the short term, but also the number of mammary cells in the long term (Henderson *et al.*, 1985; Wilde *et al.*, 1987a, 1989; Knight *et al.*, 1998).

Besides FIL, a plasmin-induced β-casein breakdown product (fraction 1-28), a potent blocker of potassium channels in mammary epithelial apical membrane, is reported to inhibit milk secretion (Silanikove *et al.*, 2000). Injection of a solution containing a casein digest enriched with β-casein fraction 1-28 into the cow's udder, or of a pure β-casein fraction 1-28 to the goat's udder, led to a transient reduction in milk yield (Silanikove *et al.*, 2000).

1.5 RESPONSES TO CHANGES IN THE FREQUENCY OF MILKING

1.5.1 Whole udder versus half-udder

Half-udder milking is advantageous to reduce between-animal variations. Moreover, it has been shown that the response to an increase in milking frequency from twice to thrice daily was not affected by the milking frequency of the opposite udder-half in cows (Morag, 1973; Hillerton *et al.*, 1990) or goats (Henderson *et al.*, 1983; 1985; Knight, 1992). Thus, little difference would be expected in percentage increases of milk yield in

response to thrice-daily milking whether frequent milking was applied to half-udder or whole-udder (Henderson *et al.*, 1983). However, Dewhurst and Knight (1994) showed that the increase of milk yield in one half-udder in response to thrice-daily milking in cows was accompanied by a small, but significant decrease of milk yield in the other half-udder, which was milked twice daily. It was suggested that the decreased milk yield might be due to a reduction in nutrient supply to the glands milked twice daily caused by the increased nutrient demand from the other glands (Dewhurst and Knight, 1994).

Nutrient repartitioning between glands within an animal is more pronounced in experiments where decreasing milking frequency or discontinuing milking in one or more glands led to compensatory increases of milk yield in untreated, control glands. Hamann and Reichmuth (1990) showed that discontinuing milking of one or more individual glands of dairy cows led to an increase of milk secretion in those glands that were still milked. In addition, the extent of the compensatory response was directly related to the number of glands that were dried off, so the greatest effect was seen when only one gland was milked. Disruption of milk secretory processes in one gland with colchicine, a plant alkaloid, also led to a compensatory response in the other gland in goats. Intramammary injection of colchicine into one gland reduced milk yield to less than 40 % of its pretreatment value, but at the same time the yield of the untreated gland increased to as high as 120 % of the pretreatment value (Henderson and Peaker, 1980; 1983a, b). Mammary extractions of glucose, acetate and most amino acids were markedly decreased on the treated gland with little changes in their arterial concentrations. It was suggested that changes of hormonal balance might be responsible for the compensatory response since the effect of additional nutrient supply from the treated gland was ruled out, based on the fact that arterial concentrations of nutrients changed little. However, without knowing the extraction rates on the untreated gland and changes of hormonal levels,

arterial concentrations alone may not be the most sensitive measure on which to exclude the effect of additional nutrient supply.

A few studies showed that decreasing milking frequency in one half-udder resulted in a compensatory increase of milk yield in the other half-udder in dairy cows (Stelwagen and Knight, 1997; Sorensen and Knight, 1999). When milking frequency was increased from two to four times daily with subsequent treatments of bST and thyroxine in both low and high genetic merit cows, milk yield was increased progressively with each treatment applied in both high and low genetic merit cows (Sorensen and Knight, 1999). Thus, the highest milk yield was obtained with the complete combination of frequent milking, bST and thyroxine. But, a further increase of milk yield was seen in the half-udder that continued on four times daily milking when milking frequency in the other half was reduced from four times to twice daily, but whole-udder milk yield remained unchanged. It was concluded that available nutrients derived from reduced milk secretion in one half of the udder might produce a compensatory increase of milk yield in the other half-udder. The compensatory response to frequent milking implies not only that mammary glands are operating below their maximum capacity, but also that the effect of frequent milking on milk production can be altered by manipulating nutrient supply. Overall, although increasing milking frequency in one half-udder does not always affect that of the other half, compensatory effects can lead to an overestimation of the response when the effects of a decrease of milking frequency are measured in half-udder rather than whole-udder.

1.5.2 Cows versus heifers

The response of milk yield to frequent milking varies between heifers and cows. In some studies, responses to frequent milking were higher in cows than in heifers (Poole, 1982; DePeters *et al.*, 1985) but, in other studies, heifers were more responsive than cows

(Amos *et al.*, 1985; Gisi *et al.*, 1986; Barnes *et al.*, 1990, Dewhurst and Knight, 1994) or responses were similar (Hillerton *et al.*, 1990; Speicher *et al.*, 1994). Observed variations of milk yield response to frequent milking were 6 – 25 % in heifers and 3 – 24 % in cows. On the other hand, Erdman and Varner (1995) reported that actual milk yield increases in response to increasing milking frequency from two to three times daily were relatively fixed at about 3.5 kg/d, regression analysis showing no significant relationships between the milk yield response to thrice-daily milking and parity or milk production during twice-daily milking. Variations of milk yield response between parities can also be found in cows milked once daily. Cows were less responsive to a reduction to once-daily milking compared with heifers (Claesson *et al.*, 1959; Davis *et al.*, 1999) or the response was similar (Rémond *et al.*, 1999). Differences between individual animals in milking potential may be one factor causing variations between experiments. Barnes *et al.* (1990) reported that three-times daily milking increased yield by 6 % during the first lactation and by very little during the second lactation in cows selected for producing high milk yield, while control cows produced 21 % and 15 % more milk, respectively, during the first and second lactations.

It might be expected that the response to frequent milking would be less in heifers than in cows because heifers are known to partition more nutrients towards their body growth (Burt, 1957; Strickland and Broster, 1981). However, there were no actual measurements of nutrient partitioning in those experiments. Although body weight changes may sometimes be a rough guide to changes of nutrient partitioning, the results are still difficult to interpret because of similar tendencies for loss of body weight in both heifers and cows in response to thrice-daily milking, with little change in food intake (DePeters *et al.*, 1985).

In contrast, cistern storage characteristics might suggest an opposite response to frequent milking between heifers and cows. It has been suggested that inconsistent responses may, in part, be due to differences in the relative cistern storage characteristics between heifers and cows (Dewhurst and Knight, 1993); cistern proportion i.e. cistern milk yield for 8 hours as a proportion of total milk yield, is larger in cows than that in heifers. Cistern proportion has been reported to be inversely related to milk yield loss during once-daily milking (Knight and Dewhurst, 1994). Because inhibition of milk secretion by FIL occurs within the alveolar lumen (Henderson and Peaker, 1987), the cistern capable of storing more milk away from the alveoli would be expected to be more effective in preventing the action of FIL (Knight *et al.*, 1995). Increased milk yield in response to continuous drainage of the cistern milk for 24 hours in cows indicates the importance of cisternal milk storage (Stelwagen *et al.*, 1996), but other results showing an absence of response to drainage of the cistern milk in goats (Henderson and Peaker, 1987) suggest that the flow of milk from the alveoli to the cistern might also be important (Davis *et al.*, 1999). Nevertheless, recent studies involving measurement of cistern proportion showed that heifers were more responsive to thrice-daily milking than were cows (Dewhurst and Knight, 1994), and that cows with large cisterns responded less to once-daily milking than those with small cisterns (Knight and Dewhurst, 1994; Stelwagen and Knight, 1997; Davis *et al.*, 1998).

1.5.3 Influence of stage of lactation

In an attempt to identify the causes of the considerable variation in response to frequent (hourly) milking in earlier work (Linzell and Peaker, 1971) where some goats showed a clear response while others showed little or no change, the responses to hourly milking in goats were reinvestigated throughout lactation in order to determine whether

responsiveness could be related to stage of lactation (Blatchford and Peaker, 1982). The lactation was divided into four periods composed of ascending, peak, declining and late lactation. Hourly milking did not affect milk secretion during peak and late lactation. Calculation of energy balance revealed that goats were in negative energy balance during peak lactation, suggesting that shortage of nutrients inhibits increase of milk secretion in response to frequent milking. Although clear evidence was not provided, it was assumed that a lack of response to hourly milking during late lactation was probably due to diversion of nutrients from mammary gland to body stores. In contrast, in a later experiment, an increase of milk yield was observed in goats milked three times daily for one week in late lactation as well as in early lactation (Henderson *et al.*, 1983). Although the same breed and similar parities of goats were used in the two experiments, the absence of detailed information on nutritional inputs makes interpretation difficult.

In a long-term experiment, thrice-daily milking of goats increased milk yield during early lactation and right through to the end of lactation (Henderson *et al.*, 1985). However, the effects of stage of lactation on the response to thrice-daily milking in the long-term experiment (mean 30 % increase) might be different from those in the short-term experiment (mean 8 % increase) since it is known that long-term thrice-daily milking increases key enzyme activities and cell number in the mammary gland (Henderson *et al.*, 1985; Wilde *et al.*, 1987a). In long-term experiments, in cows, the response to thrice-daily milking tended to be lower in early lactation than that in mid- to - late lactation (Pelissier *et al.*, 1978; Pearson *et al.*, 1979; Amos *et al.*, 1985; DePeters *et al.*, 1985). This might be partly explained by the fact that cows were in negative energy balance in early lactation (Pearson *et al.*, 1979).

Studies on the effect of stage of lactation on the response to once-daily milking are very limited. Carruthers *et al.* (1993) showed that milk yield loss in response to once-

daily milking for one week was 12 % in early lactation and 9 % in mid lactation. In another experiment, although a similar tendency for milk yield loss was observed in both stages of lactation, the response was much bigger than in the experiment of Carruthers *et al.*, milk yield loss being 38 % in early lactation and 28 % in late lactation after a three-week period of treatment (Stelwagen and Knight, 1997). Although there were differences of milk yield during twice-daily milking in these two experiments, that may not explain the variations of response because absolute milk yield has been shown to be largely unrelated to the reduction of milk yield in once-daily milking (Davis *et al.*,1999). It has been suggested that loss of milk yield in response to once-daily milking might be due to reduced activities of key mammary enzymes (Stelwagen and Knight, 1997). Since cistern proportion increases throughout lactation (Dewhurst and Knight, 1993), the results support the finding that cows with a large cistern proportion are more tolerant of once-daily milking than those with a small cistern proportion.

1.5.4 Influence of exogenous hormones

An increase of milking frequency does not affect concentrations of plasma prolactin and growth hormone in cows (Kazmer *et al.*, 1986). Interestingly, the increase of milking frequency from three times daily to six times daily (three times machine milking plus three times suckling by calf) increased markedly the plasma hormonal levels in cows in early lactation, but six times daily machine milking alone had little effect (Bar-Peled *et al.*, 1995). Experiments on the local effects of frequent milking excluded hormonal effects because the experiments used half-udder milking. Although it is worth noting that an increased milking frequency increased prolactin receptors only in glands milked more frequently (McKinnon *et al.*, 1988), prolactin is unlikely to be involved in the control of milk production in cows (Karg *et al.*, 1972; Knight, 2001).

It has been shown that the systemic galactopoietic action of exogenous bST and the local galactopoietic action of frequent milking are additive in increasing milk production in sheep (Pell *et al.*, 1989), in goats (Knight *et al.*, 1990) and in cows (Knight *et al.*, 1992; Speicher *et al.*, 1994). In a long-term experiment in goats, however, the combined treatment of bST and frequent milking resulted in a synergistic effect, lactation persistency being enhanced (Knight *et al.*, 1990).

In cows milked once daily in late lactation, milk yield was 17 % less than in those milked twice daily, but bST treatment increased milk yield by 13 %, almost overcoming the loss caused by once-daily milking (Carruthers *et al.*, 1991). Moreover, cows milked once daily in late lactation responded more to bST treatment than those milked twice daily (Stelwagen *et al.*, 1994b). One of the regulatory mechanisms operating during once-daily milking is an increase of mammary tight junction permeability, leading to a decrease of lactose concentration in milk (Davis *et al.*, 1999). Although lactose concentration in both experiments was increased in response to bST treatment, tight junction permeability was not affected by bST treatment (Stelwagen *et al.*, 1994b). Lack of capacity to store the extra milk secreted over 24 hours is unlikely to be a constraint as there was no association between 40-hour milk yield and response to bST (Carruthers *et al.*, 1991). Moreover, calculation of udder capacity based on the final udder capacity (40 hours of milk accumulation) and the average hourly secretion rate during twice-daily milking showed that the udders would be full at 25.3 hours post milking (Davis *et al.*, 1998). Therefore, it seemed likely that physical factors did not exert their maximum inhibition. It was also unlikely that bST attenuated the effects of once-daily milking, via action of FIL, on key enzyme activities of the mammary gland, because bST does not affect the mRNA and protein abundance of acetyl-CoA carboxylase and fatty acid synthase in cows (Beswick and Kennelly, 1998). Since physical capacity did not reach its

limit, the udder was able to hold more milk in response to bST during once-daily milking. But to what extent nutrient repartitioning can override chemical inhibition remains to be determined. These results, coupled with those showing compensatory responses between mammary glands, clearly suggest that mammary glands are usually operating well below their maximum capacity.

1.5.5 Influence of nutritional status

On the basis of responsiveness to hourly milking throughout lactation, it was proposed that, in declining lactation in well-fed goats, the limitation on milk secretion is not mediated by availability of nutrients at the mammary level, and that other, hormonal mechanisms may be operating (Blatchford and Peaker, 1982). It was also argued that since the stimulatory response to hourly milking was mediated locally (Linzell and Peaker, 1971), any systemic rate limitation by nutrient supply acting directly on the mammary gland would prevent the response (Blatchford and Peaker, 1982). This is supported by the finding that, with reduction in feed intake in goats in declining lactation, the response to hourly milking disappeared (Blatchford and Peaker, 1983). It is hardly surprising that considerable variation in the response of milk secretion to hourly milking was noted (Linzell, 1967; Linzell and Peaker, 1971) in studies in which no account was taken of stage of lactation and in which dietary input was not standardized. In an extreme case of dietary manipulation where energy supply was limited by fasting (Linzell, 1967) or restricting feed intake (Blatchford and Peaker, 1983), milk secretion fell markedly and no response to hourly milking was seen. However, little is known about the effects of nutrient supply, especially protein and amino acids, in association with stage of lactation, on responses to frequent milking. Less body weight gain with little change in feed intake in response to frequent milking (DePeters *et al.*, 1985; Barnes *et al.*, 1990) indicates a

possible effect of frequent milking on nutrient partitioning. Therefore, more experiments in defined dietary circumstances, in which the limiting nutrients for milk production are known, are needed if we are to understand interactions between nutrient supply and frequent milking.

Energy balance was improved in cows milked once daily during early lactation, as a consequence of the marked drop in milk yield, while food intake was maintained (Rémond *et al.*, 1999). Milking once daily caused a decrease in the plasma concentration of non-esterified fatty acids (NEFA) and an increase in glucose, suggesting improved nutritional status of the cows. In agreement with this, decreases of NEFA and increases of glucose in plasma were also observed when cows limited to 40 % of their *ad libitum* intake were switched to once-daily milking (Auldist and Prosser, 1998). There may be scope to reduce the extent of the decrease in milk yield in response to once-daily milking by changing nutrient partitioning with bST treatment and possibly also by manipulating nutrient supply (see above).

1.5.6 Responses of milk composition

In most studies, frequent milking did not affect milk composition in cows and thus yields of milk constituents increased in proportion to the increases of milk yield (Poole, 1975; Amos *et al.*, 1985; DePeters *et al.*, 1985; Gisi *et al.*, 1986; Knight *et al.*, 1992; Bar-Peled *et al.*, 1995). However, decreased milk fat (Pearson *et al.*, 1979; Barnes *et al.*, 1990, Klei *et al.*, 1997) and milk protein content (Campos *et al.*, 1994; Klei *et al.*, 1997) in response to thrice-daily milking have been reported, though fat yields and protein yields were higher than for those milked twice daily. Hillerton *et al.* (1990) reported that a small, but significant increase of milk protein content was observed in response to four times daily milking in cows. The reasons for decreased milk fat concentration are largely

unknown. In view of the positive relationship between energy status of the cow and milk protein content (Coulon and Rémond, 1991), it might be expected that frequent milking would be likely to reduce milk protein content since cows in long-term trials of frequent milking gained less body weight with little change in feed intake, relative to those milked twice daily. Although this view appears not to be supported by most long-term trials, it should be noted that there is little information regarding dietary input and body weight changes in those experiments that show decreased or increased milk protein content in response to thrice-daily milking, and this makes interpretation difficult. Nevertheless, the effect of frequent milking on milk protein content has led to a more detailed analysis of protein composition because frequent milking reduces exposure of milk protein to proteolytic enzymes as a result of decreased storage time of milk in udder (Klei *et al.*, 1997). There is a suggestion that casein as a proportion of total protein was increased in response to thrice-daily milking in mid lactation, with little changes in plasmin activity throughout lactation (Klei *et al.*, 1997). However, a more recent study showed that, in long-term half-udder thrice-daily milking, casein as a proportion of total protein was higher in thrice-daily milking than that in twice-daily milking, and that three times daily milking decreased plasmin in milk (Sorensen *et al.*, 2001). The ratio of sodium to potassium was decreased, and lactose content was increased in thrice-daily milking compared with twice-daily milking, suggesting that mammary tight junctions became tighter in response to thrice-daily milking.

In general, once-daily milking increases fat and protein content, and decreases lactose content (Carruthers *et al.*, 1991; Holmes *et al.*, 1992; Stelwagen *et al.*, 1994b; Rémond *et al.*, 1999; Davis *et al.*, 1999). Many of the changes in milk composition may be ascribed to changes in the permeability of the tight junctions between the secretory cells, leading to increased exchange of milk and interstitial fluid i.e., most notably,

increased serum albumin and decreased lactose content in milk. An increase of proteolytic enzymes in milk has also been reported during once-daily milking (Stelwagen *et al.*, 1994c). A small increase in the concentration of casein may also contribute to the higher total milk protein content (Claesson *et al.*, 1959; Rémond *et al.*, 1999), which might be related to an improved energy status in cows. Nevertheless, the ratio of casein to whey protein is reduced in response to once-daily milking (Auldist and Prosser, 1998; Lacy-Hulbert *et al.*, 1999).

1.6 CONCLUSIONS

It is clear that, in some circumstances, dairy cows increase milk yield in response to improvements in nutrient supply from the diet, and that the increases in milk secretion occur in response to improvements in the total supply of energy-yielding nutrients and to more specific increases in the supply of amino acids. It is equally clear that, in some circumstances, cows increase milk yield in response to an increase of milking frequency. However, for both types of response there are wide variations between experiments. For the nutritional responses, it is likely that the variation reflects our incomplete knowledge of the detailed nutritional circumstances and of the physiological status of the animals. In experiments on frequency of milking, nutrition has rarely been taken into account, and even then, it has been in only the most general way.

The result is that we have little or no knowledge of interactions between the two types of effect. An example from the recent experiments illustrates the problem. In cows in which milk yield is limited by a deficiency in the supply of specific amino acids, milk yield increases markedly in response to intravenous infusions of the limiting amino acids within 24 hours (Kim *et al.*, 1999; 2000). Presumably, when amino acids were limiting milk secretion, the local inhibition of secretion by FIL did not occur. This would suggest

that there would have been no response to an increase of milking frequency, unless the response occurred by a separate mechanism. On the other hand, things may not be that simple. The cows in these experiments consumed ME well in excess of their requirements and it would seem that the only limitation on the synthesis of milk constituents was for milk protein. Might they then have responded to increased milking frequency by increasing milk yield but reducing the concentration of milk protein?

Answers to such questions are important because we need to know how limitations on milk secretion at a local level within the mammary gland relate to the nutritional status of the animal herself. We need to know to what extent nutritional deficiencies can override, or modify, responses to changes in milking frequency. In what circumstances, if any, can an increase in milking frequency alter nutrient partitioning even to the extent of causing a net mobilization of body tissue? Might a combination of manipulations of milking frequency and nutrition be used to manipulate the composition of milk?

1.7 AIMS AND OBJECTIVES

The work described in this thesis was aimed at providing information on interactions between responses to increased frequency of milking and nutrition. Rather than considering nutrition in the general sense of the total supply of nutrients, attention was focused on the effects of deficiencies in the supply of specific amino acids. This had the advantage of building on the foundations of the results of a series of investigations carried out at this Institute over the last five years or so. This allowed the nutritional circumstances within the experiments reported here to be clearly defined in relation to amino acid status.

An initial experiment to establish the pattern of response with time to differences in amino acid supply was followed by more specific investigations of response to increases in milking frequency when a specific group of amino acids was either deficient or supplied in excess. To explore effects of milking frequency on nutrient partitioning, bST was used in one experiment as a repartitioning agent.

CHAPTER TWO

MATERIALS AND METHODS

2.1 ANIMALS

Diets and protocols regarding the experimental animals are described in subsequent chapters.

2.1.1 Management of animals in the experiments

The cows were housed individually in metabolism stalls with free access to drinking water and fed twice a day in the experiments of Chapter 3. Feed intake was determined daily prior to the afternoon milking and the silage offered was adjusted to ensure a refusal of approximately 15 % of that offered. For the rest of the studies, cows were housed together in a metabolism cubicle with free access to drinking water and fed using an automatic feeder (RIC HF 2PL, Insentec B.V., Marknesse, Netherlands). Feed intake was controlled and monitored through a computer connected to the automatic feeder. Milking regimes in all studies are described in their respective chapters.

2.2 COLLECTION AND PREPARATION OF SAMPLES

2.2.1 Feedstuffs

Silage samples were taken from the silo every two weeks and a subsample of 1,500 g was dried and ground through a 1 mm screen for analysis. Another subsample of 1,500 g was minced through a 100 mm dye (Crypto Ltd., London, UK) and stored frozen until analysed.

All other feedstuffs were also sampled every two weeks. 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 until analysed.

2.2.2 Milk

Milk samples were collected from the last four consecutive milkings of each treatment period. Milk samples 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 analysed. At the end of each sampling period, milk samples from individual cows were warmed to 40 °C in a water bath to disperse the fat globules and then bulked according to milk yield.

2.2.3 Blood

Samples of blood were withdrawn from a tail vessel into heparinized vacutainer tubes (Becton and Dickinson, Vacutainer System Europe, Meylan-Cedix, France) through a small-bore needle (20G) while animals were restrained in stalls. Samples were centrifuged at 1,500 g for 15 minutes and the blood plasma was removed and stored immediately at -20 °C until analysed.

2.2.4 Urine

Total urine was collected via bladder catheter (silicone treated latex Foley catheter, 28Ch, 2-way male, 30 ml balloon, Folec, Malaysia) in a preliminary experiment in Chapter 4. After the catheter was inserted into the bladder, 50 ml of saline was injected into the catheter to inflate a balloon in order to secure the catheter in the bladder. Five metres of silicone tubing was connected to the catheter to collect urine into a plastic

container which contained 250 ml of 5 M sulphuric acid to keep pH less than 3. To collect spot urine samples cows were induced to urinate by vulval stimulation or by inserting a bladder catheter. Spot urine collections were performed once at the end of each treatment period. Three ml of 5 M sulphuric acid were added to 35 ml of spot urine to keep pH less than 3. A representative portion of urine was stored at -20 °C until analysed.

2.2.5 Mammary tissue biopsy and udder volume

Biopsy

Mammary tissue collection was performed using a modification of the method of Knight *et al.* (1992a). Cows were milked prior to biopsy with the aid of 20 units of oxytocin (intra-muscular, Intervet UK Ltd., Buckinghamshire, UK). The procedure was performed in a crush with the cow in a standing position. The left side of the udder was clipped and then sterilized with Hibitane solution containing 10 % (v/v) Hibitane (5 % concentrate, v/v; Zeneca Ltd., Macclesfield, Cheshire, UK), 75 % (v/v) ethanol and 15 % (v/v) distilled water. Biopsy sites were selected in the basal (upper) portion of the udder by palpation, avoiding fat and larger subcutaneous blood vessels. Local anaesthesia was achieved by a line block subcutaneous injection of 5 ml 2 % lignocaine hydrochloride (Lignocaine & Adrenaline, Norbrook Laboratories Ltd., Newry, UK). For the insertion of a biopsy needle (Core Tissue Biopsy Needle, Length of Sample Notch: 9 cm, C. R. Bard Inc., Covington, USA) connected to a biopsy instrument (C. R. Bard Inc.), a 1 cm incision was made through the skin using a scalpel. A 50 – 100 mg portion of secretory tissue was collected. Haemostasis was achieved with temporary packing with dry swabs and then chlortetracycline hydrochloride power (Aureomycin, Cyanamid, Hampshire, UK) was applied to the incision site. A syringe of antibiotic containing 200 mg cloxacillin and 75 mg ampicillin (Kloxerate Plus, Fort Dodge Animal Health Ltd., Southampton, UK) was

injected through the teat canal of the biopsy udder to prevent any infection. A portion of mammary tissue was kept in a 2-ml screw-capped plastic tube and stored in liquid nitrogen immediately after tissue removal for the determination of key enzyme activities and DNA content. A further portion of tissue was placed in fixative (4 % formalin, pH 7.4) for the determination of PCNA.

Udder volume

The volume of the empty udder was determined by the method of Knight and Dewhurst (1994). Cows were milked prior to the measurement with the aid of 20 units of oxytocin and then restrained in a crush with the hind legs tied apart. The udder and surrounding skin, which had previously been clipped, were coated liberally with udder cream and the udder was spray-coated with a quick-setting polyurethane foam (Froth pack 180, Foampax Scotland, Newmilns, UK), which became fully rigid within 1 – 2 min, forming a cast conforming to the shape of udder. The volume of the cast was determined by filling with barley of known specific gravity.

2.3 ANALYTICAL METHODS

2.3.1 Dry matter and ash

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

2.3.2 pH of silage

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

2.3.3 Total nitrogen

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

2.3.4 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.

Reagent: Tannic acid solution was prepared by dissolving 4.45 g tannic acid in water and adding 0.1 ml of concentrated sulphuric acid. The mixture was made up to 100 ml with distilled water. This was allowed to stand for 24 hours and then filtered through Whatman No. 42 filter paper.

Procedure: Wet minced silage (1 g) was weighed accurately into a centrifuge tube and 20 ml boiling tannic acid solution were added. The tube was placed in a boiling water bath for 15 minutes, cooled for 15 minutes and then centrifuged at 1,500 g for 10 minutes. The supernatant was removed by suction through a tube covered at the end with a layer of fine, washed muslin. Any particles of sample were washed from the muslin back into the tube and the volume made up to 25 ml with distilled water. The residue was resuspended and the tube centrifuged as before. The washing and centrifuging was repeated twice more and the residue was finally washed into a Kjeldahl digestion tube for nitrogen determination.

2.3.5 Ammonia nitrogen in silage

This was determined on a water extract of the sample. The extract 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 filtered by squeezing the silage juice through muslin and then centrifuged at 1,500 g for 20 minutes. Ten ml of silage extract were pipetted into a Kjeldahl digestion tube and placed in the Kjeltec 1030 analyser, and the ammonia released after addition of NaOH was absorbed in 4 % (w/v) boric acid solution and titrated with 0.02 M HCl using basic acid indicator solution containing bromocresol green/methyl red.

2.3.6 Lactic acid in silage

Lactic acid was determined by the method of Elsden and Gibson (1954) in which lactic acid is oxidized 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.

Reagents:

1) 2 % (w/v) ceric sulphate solution in 0.5 M sulphuric acid.
2) 0.05 M iodine solution, prepared by dissolving 20 g potassium iodide in 35 ml water and then dissolving 13 g of iodine in this solution and making up to 1 l with water.

Procedure: 9.5 ml silage extract were transferred into a 15-ml centrifuge tube and 0.5 ml copper sulphate and 1 g calcium hydroxide were added. These were mixed and allowed to stand for 30 minutes and then centrifuged until clear. One ml of the clear solution was transferred into a 100-ml flask with 0.5 ml 5 M sulphuric acid and a few anti-bumping granules and steam distillation was commenced, 5 ml ceric sulphate being added through

the separating funnel. Fifteen ml of distillate were collected in a 50-ml conical flask containing 2 ml of 0.5 % (w/v) sodium metabisulphate. One ml of 2 % (w/v) starch solution and 0.05 M iodine were added until a permanent blue colour was obtained and then decolourization was achieved by adding 0.1 M sodium thiosulphate. After regaining the blue colour with 5 mM iodine, 1 g of sodium hydrogen carbonate was added and then titrated with 5 mM iodine.

2.3.7 Total soluble sugars in silage

These were determined by a method similar to that of Somogyi (1945).

Reagents:

1) Arsenomolybdate reagent, prepared by dissolving 25 g ammonium molybdate in 450 ml water and then adding 21 ml concentrated sulphuric acid. A solution of 3 g (12 %) di-sodium hydrogen arsenate in 25 ml water was added in a water bath at 55 °C for 25 minutes with continuous stirring. The mixture was transferred to a brown bottle, incubated for 24 hours at 37 °C, cooled and held at 4 °C until used.

2) Reagent A, B and C

i) Reagent A, prepared by dissolving 25 g sodium carbonate, 25 g Rochelle salt (potassium sodium tartrate) and 20 g anhydrous sodium sulphate in 800 ml water and diluting to 1 l.

ii) Reagent B, 15 % (w/v) copper sulphate solution containing 1 or 2 drops of concentrated sulphuric acid per 100 ml.

iii) Reagent C, reagents A and B were made up as 1 part B to 25 parts A.

Procedure: A sample (5 ml) of silage extract was pipetted into a glass stoppered tube for hydrolysis. 0.1 ml of 0.5 M sulphuric acid was added and the tube and its contents were boiled in a boiling water bath for 30 minutes. The tubes were cooled in a water bath and

then 0.1 ml of 1 M NaOH was added. Two ml of hydrolysate were transferred to duplicated 15-ml centrifuge tubes and deproteinized by adding 4 ml of 5 % (w/v) zinc sulphate solution and 4 ml of 0.3 M sodium hydroxide. After mixing, the contents of the tube were centrifuged at 1,500 g for 10 minutes. Two ml of supernatant or standard were transferred to a glass-stoppered tube containing 2 ml of reagent C. The tube was heated in a boiling water bath for 10 minutes. After cooling 2 ml of arsenomolybdate reagent were added, the solution transferred to a 50-ml volumetric flask and made up to volume with water. The absorbance was read on a spectrophotometer (Cecil instruments, Cambridge, UK) at 500 nm against a blank of distilled water. The total soluble sugars in samples were calculated by reference to a calibration graph derived from standard solution of D-glucose containing 50 to 250 ml/l.

2.3.8 Ethanol in silage

Ethanol was determined by gas chromatography by the method of Huida (1982) using methanol as an internal standard. Thirty ml of dry methanol were added to 5 ml of silage extract and 1 µl injected onto the column of a Shimadzu GC-8A gas chromatograph (Shimadzu Europe Ltd, Milton Keynes, Buckinghamshire, UK) fitted with a flame ionization detector. The column 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 was 60 ml/min.

2.3.9 Total and individual volatile fatty acids (VFA) in silage

The VFA in the silage were determined by gas chromatography by the procedure of Cottyn and Boucque (1968).

Reagents:

1) Preservative mixture, containing 30 ml metaphosphoric acid (25 % w/v), 10 ml formic acid (90 % w/v) and 20 ml distilled water.
2) Internal standard, hexanoic acid (2 g) dissolved in 1 l distilled water.
3) VFA standard solution, prepared by pipetting 4 ml acetic acid (6 g/100 ml water), 2 ml propionic acid (7.2 g/100 ml), 2 ml butyric acid (8.4 g/100 ml), 2 ml isobutyric acid (0.8 g/100 ml), 2 ml valeric acid (0.96 g/ 100 ml) and 2 ml isovaleric acid (0.96 g/100ml) into a 100 ml volumetric flask and diluting to volume with water.

Procedure: Two ml of silage extract were transferred to a 10 ml test tube and 1 ml preservative and 2 ml hexanoic acid were added and the contents mixed well. The tube was shaken and allowed to stand for 20 minutes and then centrifuged at 1500 g for 20 minutes. The supernatant was analysed using a Shimadzu GC-8A gas chromatograph. The sample (1 – 3 µl) was injected onto 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 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.

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

The NDF and ADF contents in food were determined by the method of Goering and Van Soest (1970).

Reagents:

1) Neutral detergent (ND) solution contained 30 g sodium lauryl sulphate, 18.6 g disodium ethylenediaminetetraacetic acid dihydrate, 6.81 g sodium borate

decahydrate, 4.56 g anhydrous disodium hydrogen phosphate and 10 ml 2-ethoxyethanol in 1 l of solution.

2) Acid detergent (AD) solution consisted of 20 g cetyl-trimethyl ammonium bromide per l of 0.5 M sulphuric acid.

Procedure: Approximately 1 g of sample was added to a 500 ml round-bottom flask to which 100 ml of ND solution, 2 ml Dekalin and 0.5 g sodium sulphate for NDF determination or 100 ml AD solution and 2 ml Dekalin for ADF determination were added. The flask was then refluxed for 60 minutes after the onset of boiling and the contents were then transferred to a pre-weighed sintered glass crucible (porosity 1) which had been previously set on a filter manifold. The flask and the inside of the crucible were washed twice with boiling water and twice with acetone. The crucible and its contents were then dried overnight in an oven at 100 °C and re-weighed after cooling in a desiccator. The sample was then ashed at 580 °C for 3 hours. The ash-free concentrations of NDF and ADF were reported as the loss in weight on ashing.

2.3.11 Digestible organic matter in the dry matter (DOMD or 'D' value) in silage

The estimation of 'D' value of dried silage samples was based on their lignin content which was analysed using the method of Morrison (1972).

Reagents:

1) Acetyl bromide-acetic acid reagent, prepared by transferring 25 ml acetyl bromide into a 100-ml volumetric flask and then making up to volume with glacial acetic acid.

2) 0.5 M hydroxylammonium chloride solution.

Procedure: 50 mg of dried silage was weighed accurately into a 25-ml Quickfit tube and 20 ml of water added. The tube was stoppered, mixed and heated at 70 °C in a water bath for 30 minutes. The tube was shaken at 10-minute intervals. The sample was then filtered

through a Whatman No. 52 filter paper and washed in order with water, ethanol, acetone and diethyl ether. The filter paper and sample were transferred back to the tube and all traces of organic solvent removed in the oven at 47 °C overnight. To the residue in the tube was added 5 ml of acetyl bromide-acetic acid reagent. The tube was shaken, stoppered and heated in a water bath at 70 °C for 30 minutes with shaking as before. After allowing the tube to cool in a water bath at 20 °C for 30 minutes, 20 ml of glacial acetic acid were added and mixed. A 5 ml aliquot was transferred to a 50-ml volumetric flask to which was also added 7.5 ml of glacial acetic acid and 1 ml of 2 M sodium hydroxide. The volume was made up to approximately 45 ml with ethanol, 1.5 ml hydroxylammonium chloride was added, the flask shaken and contents made up to volume with ethanol. The flask was shaken again and allowed to stand for 1 hour before the contents were filtered through a Whatman No. 52 filter paper. A blank was prepared as above without the addition of sample. Lignin is estimated as an 'A' value by measurement of the optical density at 280 nm.

Calculation:

The lignin content was reported as an 'A' value from the following formula:

'A' = $OD_s - OD_b$ / C litre/g/cm

where OD_s = optical density of sample

OD_b = optical density of blank

C = weight of sample dry matter x 4

The 'D' value of the silage was calculated using the following regression equation:

'D' = 86.148 – 10.907 A

2.3.12 Milk fat, lactose and protein

Milk fat, lactose and protein contents were analysed by Scottish Milk Laboratories Ltd. (Paisley, UK) using infrared analysis.

2.3.13 Fatty acid composition of milk fat

Fat was extracted from milk by the method of Bligh and Dyer (1959) and methyl esters were formed by a procedure modified from that of Christoperson and Glass (1969).

Procedure: The sample of milk was heated slowly to 40 °C in a water bath and shaken to disperse the fat globules. One ml of milk was pipetted into a glass-stoppered 50-ml tube containing 3 ml water. Ten ml methanol and 5 ml chloroform were added and mixed well. A further 5 ml chloroform were added and mixed again. Five ml of 0.88 % (w/v) KCl were added and, after mixing, the mixture was centrifuged at 1,500 g for 15 minutes. The lower layer was transferred, using a Pasteur pipette, to a 50 ml round-bottomed flask and evaporated to dryness at 50 – 60 °C using a rotary evaporator. The extracted lipid was washed with acetone and redried. The lipid was taken up in 3 ml hexane and transferred to a 15-ml conical test tube. The flask was washed with another 3 ml hexane and the washings were added to the test tube. The hexane was evaporated from the sample at 50 – 60 °C under nitrogen and 0.5 ml hexane and 0.02 ml of 2 M sodium methoxide in dry methanol was added to methylate the lipid. The tube was stoppered and shaken at room temperature for 5 minutes. Hexane (0.5 ml) and a little dry calcium chloride powder were added. The sample was allowed to stand for 2 – 3 minutes. The supernatant was poured off into a stoppered glass tube and stored at 4 °C until analysed by gas chromatography.

The methyl esters of the fatty acids were separated on a glass column packed with 15 % EGSS-X on Gas Chrom P 100/120 mesh (Pierce and Warriner Ltd., Chester) using a temperature programmed run. The initial temperature of 80 °C was held for 4 minutes

after which the temperature was increased at a rate of 6 °C/min up to 180 °C. The nitrogen carrier gas flow rate was 40 ml/min.

The proportion of each fatty acid in the mixture was calculated from its peak area relative to that of palmitic acid. Corrections were made for differences in the response of the detector to each acid using factors derived from the analysis of a standard mixture of known amounts of caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acids.

2.3.14 Determination of individual amino acids

The AA composition of blood plasma was determined by a modified version of the method of Umagat *et al.* (1982) using high-performance liquid chromatography (HPLC) with ortho-phthaldialdehyde/2-mercaptoethanol (OPA/MCE) precolumn derivatization.

The OPA/MCE reagent reacts with the primary amine function of the AA to form a fluorescent 1-alkyl-thio-2-alkyl-substituted isoinodol. The OPA reaction is specific for primary amines and HPLC analysis of the OPA/amine derivatives permits high sensitivity of detection and offers the advantages of relatively short analytical run times and no interference from ammonia.

Apparatus: The HPLC system consisted of a Spectra-Series P200 solvent delivery system (Thermo Quest, Herts, UK) coupled to a Gilson Model 121 filter fluorimeter (Anachem Ltd., Luton, Bedfordshire, UK) with wavelength of 305-309 nm excitation filter and a 430 – 470 nm emission filter. Separations were carried out on a 250 x 4.6 mm ID Apex II column prepacked with 5 μm octadecyl particles (Jones Chromatography, Hengoed, UK). Sample injections were made using a Gilson Model 401 sample dilutor and a Gilson Model 231 sample injector. The chromatographic data were processed by a Shimadzu C-R1B integrator.

Reagents:

1) OPA/MCE derivatizing reagent, prepared by dissolving 125 mg OPA with 2.5 ml HPLC grade methanol in a 25-ml volumetric flask and then made up to volume with 0.4 M sodium borate buffer (pH 9.5). One hundred microlitres of MCE were added to the solution and stored in dark and allowed to stand for 24 hour before use. Every day 10 µl of 2-mercaptoethanol were added.
2) 0.4 M sodium borate buffer (pH 9.5), made by dissolving 24.732 g boric acid in 970 ml water and pH adjusted to 9.5 with 4 M NaOH. The solution was made up to 1 l with water and filtered through 0.45 µm pore size Whatman filter paper.
3) 6 M HCl solution, prepared by adding 501 ml concentrated HCl (specific gravity 1.18) to 499 ml water and then 0.5 ml of 2-mercaptoethanol was added.
4) Citrate buffer (pH 2.2), prepared by dissolving 19.6 g sodium citrate in 700 ml water and adding 16.5 ml concentrated HCl, 20 ml thiodiglycol, 2 ml Brij-35 solution and 0.1 ml octanoic acid and making up to 1 l with water.
5) Solvent A was 0.05 M sodium acetate (pH 5.7), HPLC tetrahydrofuran and HPLC grade acetonitrile in a ratio of 96: 1: 3.
6) Solvent B was HPLC grade methanol.
7) AA standard, prepared by adding asparagine, ornithine, taurine, α-aminobutyric acid, γ-aminobutyric acid and tryptophan solution to the commercial Sigma standard (AA-S-18) for plasma analysis. The concentration of standard was 500 µM and the standard was kept at – 20 °C until analysed.

Preparation of plasma samples

Plasma samples were deproteinized using 5-sulphosalicylic acid (SSA). Equal volumes of plasma and SSA were mixed in the centrifuge tube and centrifuged at 4 °C for

30 minutes at 1,500 g. One ml of supernatant was added to 1 ml of internal standard (homoserine 200 μM). This mixture was filtered through a 0.2 μm syringe filter.

Chromatographic conditions: OPA/MCE derivatization was conducted in the dilutor at room temperature and 20 μl of the derivatized mixture was injected on to the column by the automatic sample injector. The solvents used were degassed with degasser (SCM 1000, Thermo separation products, FL, USA) throughout the analysis and the gradient program was applied (Table 2.1). The flow rate was maintained at 1 ml/min. Analytical run time was 42 minutes.

Procedure: The AAs in deproteinized plasma samples were separated by elution with solvents in order of hydrophobicity. Identification of the individual AAs was made by reference to their retention times measured under the given conditions when standard mixture of AAs was analysed. A typical chromatogram of AA standard for plasma analysis is shown in Figure 2.1.

2.3.15 Plasma albumin

The concentration of albumin in blood plasma was determined using a commercial kit (Sigma Diagnostics, St. Louis, MO, USA). Albumin binds to bromocresol green reagent (BCG) to produce a blue-green colour with an absorbance maximum at 628 nm. The intensity of the colour produced is directly proportional to albumin concentration.

2.3.16 Plasma glucose

The concentration of glucose in blood plasma was determined using a commercial kit (Boehringer Mannheim GmbH, Germany). The glucose is oxidized by glucose oxidase and, in the presence of peroxidase, the hydrogen peroxide form the oxidized chromogen,

Table 2.1 Chromatographic gradient conditions for HPLC analysis of amino acids

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)
0	90	10	1
2	90	10	1
3	80	20	1
25	55	45	1
40	20	80	1
42	20	80	1
43	0	100	1
50	0	100	1

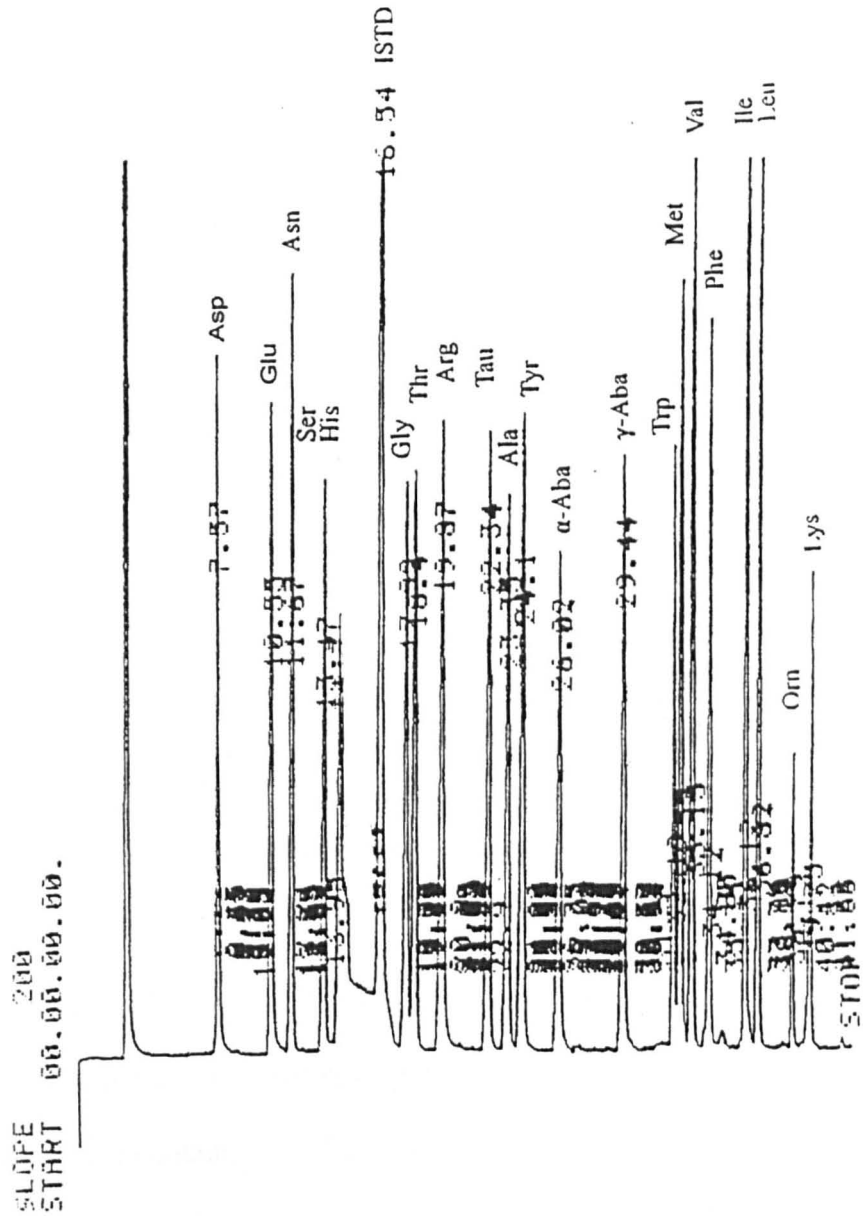


Figure 2.1 Chromatogram of an amino acid standard for plasma analysis (Asp, aspartic acid; Glu, glutamic acid; Asn, asparagine; Ser, serine; His, histidine; ISTD, internal standard; Gly, glycine; Thr, threonine; Arg, arginine; Tau, taurine; Ala, alanine; Tyr, tyrosine; α-Aba, α-aminobutyric acid; γ-Aba, γ-aminobutyric acid; Trp, tryptophan; Met, methionine; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Orn, ornithine; Lys, lysine).

Perid (2,2 Azino-di[3 ethyl-benzthiazoline sulphonate]). The colour intensity of the dye is proportional to the glucose concentration.

2.3.17 Plasma non-esterified fatty acids (NEFA)

The concentration of NEFA in blood plasma was determined by the method of Itaya and Ui (1965). The NEFA dissolved in chloroform forms Cu complex in the presence of Cu-triethanolamine. The trace amount of Cu complex in the chloroform layer reacts with sodium diethyldithiocarbamate. The intensity of the colour produced is proportional to NEFA concentration.

Reagents:

1) 6.45 % (w/v) $Cu(NO_3)_2 \cdot 3H_2O$ in a ratio of 9: 1: 10(v/v/v). The mixture was stored at 4 °C and used within 5 days.
2) 0.1 % (w/v) sodium diethyldithiocarbamate solution (SDC), prepared by dissolving 0.05 g of sodium diethyldithiocarbamate in 50 ml butanol-1-ol.
3) 10 mM palmitic acid, prepared by dissolving 0.256 g palmitic acid in 100 ml chloroform.

Procedure: The standard was prepared by diluting 20 µl of 10 mM palmitic acid with 0.3 ml water. Three-hundred microlitres of plasma or standard were added to a 10-ml screw-capped plastic tube containing 2.25 ml chloroform and 1 ml of 0.01 M sodium phosphate buffer (pH 6.5). The mixture was shaken for 120 seconds and allowed to stand for 30 minutes at room temperature. The upper layer was aspirated with a Pasteur pipette and 1.5 ml of Cu mixture added, shaken for 90 seconds and centrifuged at 1,500 g for 15 minutes. The upper layer was aspirated with a Pasteur pipette and 1 ml of the residual chloroform layer was transferred into a glass tube. After addition of 50 µl SDC, the contents of the tube were mixed well and absorbance measured spectrophotometrically at 440 nm. After

correction for the blank (water), the absorbance of the sample was divided by that of standard.

2.3.18 Plasma urea

Determination of the urea concentration in blood plasma was carried out using a commercial kit (Sigma Diagnostics). Urea is hydrolysed by urease to ammonia and carbon dioxide. Ammonia then reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside, to form indophenol. The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrophotometrically at 570 nm.

2.3.19 Total haemoglobin in blood

The concentration of total haemoglobin in blood was determined using a commercial kit (Sigma Diagnostics). The haemoglobin was oxidized to methaemoglobin in the presence of alkaline potassium ferricyannide. Methaemoglobin reacts with potassium cyanide to form cyanmethaemoglobin which has maximum absorption at 540 nm. The colour intensity measured at 540 nm is proportional to the total haemoglobin concentration.

2.3.20 Creatinine in urine

Creatinine in urine was determined by the method of Shingfield and Offer (1999) using HPLC under isocratic conditions.

Apparatus: The HPLC system used consisted of Gilson 305 and 306 pumps, a Gilson 811C Dynamic mixer and a Gilson 805 manometric module coupled to a Gilson 115 UV detector with wavelength of 190 - 380 nm. Separations were carried out on a 5 μm

Spherisorb ODS II C_{18} reversed-phase column (250 x 4.6 mm I.D.; Waters, Milford, MA, USA) without the use of a precolumn. Sample injections were made using a Gilson 234 Autoinjector. The chromatographic data were processed by an SP4400 integrator (Thermo separation products).

Reagents:

1) Mobile phase (pH 3.0), prepared by dissolving 2.02 g sodium 1-heptane sulphonic acid and 0.86 g ammonium dihydrogen phosphate and 0.14 ml of triethylamine in a 1 l volumetric flask and making up to volume with double-deionized water. This mixture was filtered through 0.45 μm pore size Whatman filter paper.
2) Urine diluent (pH 2.1), made by using the same procedure as described for the mobile phase with the exception that triethylamine was omitted.
3) The stock standard solution (300 μM), prepared by dissolving 67.86 mg of creatinine in 2 l of urine diluent. Additional working standards were prepared by diluting the stock standard 50 % (v/v) and 25 % (v/v) with urine diluent.

Preparation of samples: Urine samples were centrifuged for 5 minutes at 3,300 g. One ml of supernatant was transferred into a 50-ml volumetric flask and then made up to volume with urine diluent and mixed. A 2 ml aliquot of this mixture was filtered through a 13 mm syringe filter with 0.45 μm Whatman cellulose nitrate membrane.

Chromatographic conditions: Chromatography was achieved under isocratic conditions at a flow rate of 1.0 ml/min. Twenty microlitres of sample were injected on to the column by the automatic sample injector. The mobile phase was degassed with helium throughout the analysis. Separation was achieved at 20 °C with a total run time of 60 minutes. Eluted mobile phase was monitored at 218 nm. Column regeneration was performed by washing with the following solutions in sequence: distilled water, 50 % (v/v) aqueous acetonitrile, 100 % acetonitrile, 50 % (v/v) aqueous acetonitrile, distilled, deionized water.

Procedure: Creatinine was identified by its retention time and co-elution with standard. The creatinine was calculated by reference to a calibration graph derived from standard solutions of creatinine containing 75 – 300 μmol/l. A chromatogram of a creatinine standard for urine analysis is shown in Figure 2.2.

2.3.21 3-methylhistidine in urine

The concentration of 3-methylhistidine in urine was determined by the method of Wassner *et al.* (1980) using HPLC with fluorescamine precolumn derivatization.

Apparatus: The HPLC system, a sample injector and an integrator used were as described for creatinine with the exception that the UV detector was replaced with a Gilson Model 121 filter fluorimeter. Separations were carried out on a 250 x 4.6 mm ID Apex I column prepacked with 5 μm octadecyl particles (Jones Chromatography).

Reagents:

1) Fluorescamine derivatizing reagent, prepared by dissolving 40 mg fluorescamine in 25 ml acetonitrile. This was stored in a dark bottle.
2) 0.4 M sodium borate solution (pH 12.2) containing 10 μmol/ml of histidinol (internal standard), prepared by dissolving 2.473 g of boric acid in 80 ml water and adding 0.214 g of L-histidinol dihydrochloride. This solution was made up to 100 ml with distilled water.
3) 0.5 M morpholino propane sulphonic acid (MOPS), prepared by dissolving 10.465 g MOPS in 100 ml of 3 M NaOH.
4) Solvent A, 10 mM sodium phosphate (pH 7.5).
5) Solvent B, HPLC grade acetonitrile.

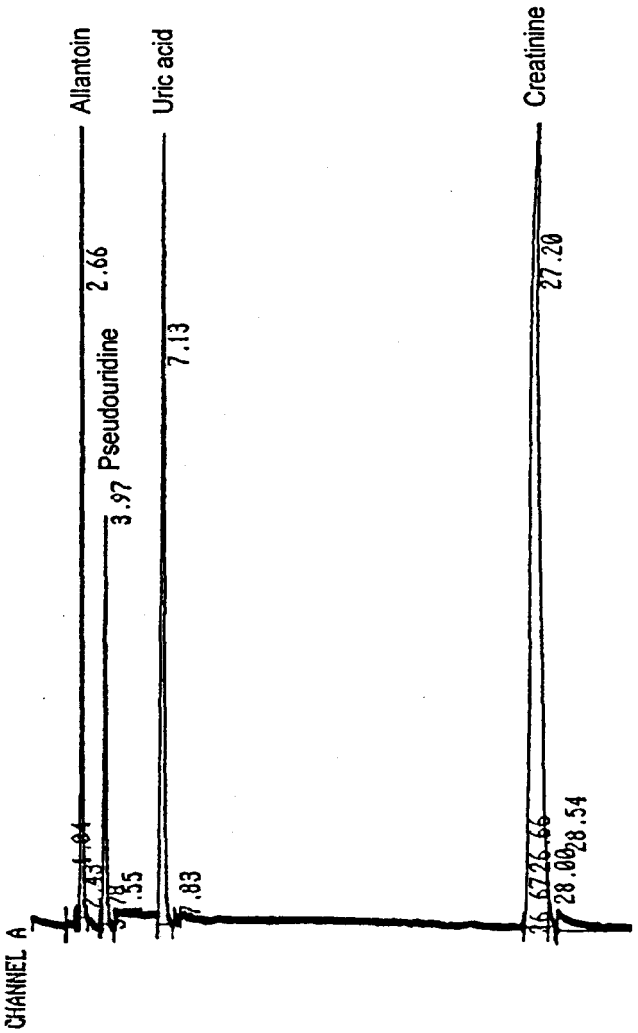


Figure 2.2 Chromatogram of a creatinine standard for urine analysis.

6) 3-methylhistidine standard (10 μM), prepared by dissolving 8.46 mg 3-methylhistidine in 100 ml of 0.01 M HCl. A 1 ml aliquot was transferred into a 50-ml volumetric flask and then made up to volume with 0.01 M HCl.

Preparation of samples: Urine was diluted 1: 2 with distilled water. Two hundred microlitres of diluted urine were added to an Eppendorf tube containing 750 μl water and 50 μl of 70 % perchloric acid. The tube was mixed and then centrifuged at 14,000 g for 1 minute. One hundred- and -fifty microlitres of standard or supernatant of urine were placed in a 1.5-ml screw-capped plastic microtube along with 500 μl of 0.4 M sodium borate solution containing 10 μmol/ml of histidinol and then 500 μl of fluorescamine derivatizing reagent were added. The tube was mixed well and then allowed to stand for 5 minutes. Seventy microlitres of concentrated perchloric acid were added to the tubes, which were capped, heated at 80 °C for 1 hour in a heating module (Pierce, Rockford, IL, USA), cooled to room temperature, and acid neutralized with 200 μl of 0.5 M MOPS.

Chromatographic conditions: One-hundred- and -fifteen microlitres of the derivatized mixture were injected on to the column by the automatic sample injector. The solvents used were degassed with helium throughout the analysis and the gradient program was applied (Table 2.2). The flow rate was maintained at 1.5 ml/min. Analytical time was 45 minutes.

Procedure: Identification of 3-methylhistidine was made by reference to its retention time measured under the given conditions when a 3-methylhistidine standard was analysed. Chromatogram of a 3-methylhistidine standard for urine analysis is shown in Figure 2.3.

2.3.22 Growth hormone (GH) radioimmunoassay (RIA)

The growth hormone assay was based on the method described by Vernon *et al.* (1981).

Table 2.2 Chromatographic gradient conditions for HPLC analysis of 3-methylhistidine

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)
0	80	20	1.5
30	75	25	1.5
35	20	80	1.5
45	20	80	1.5

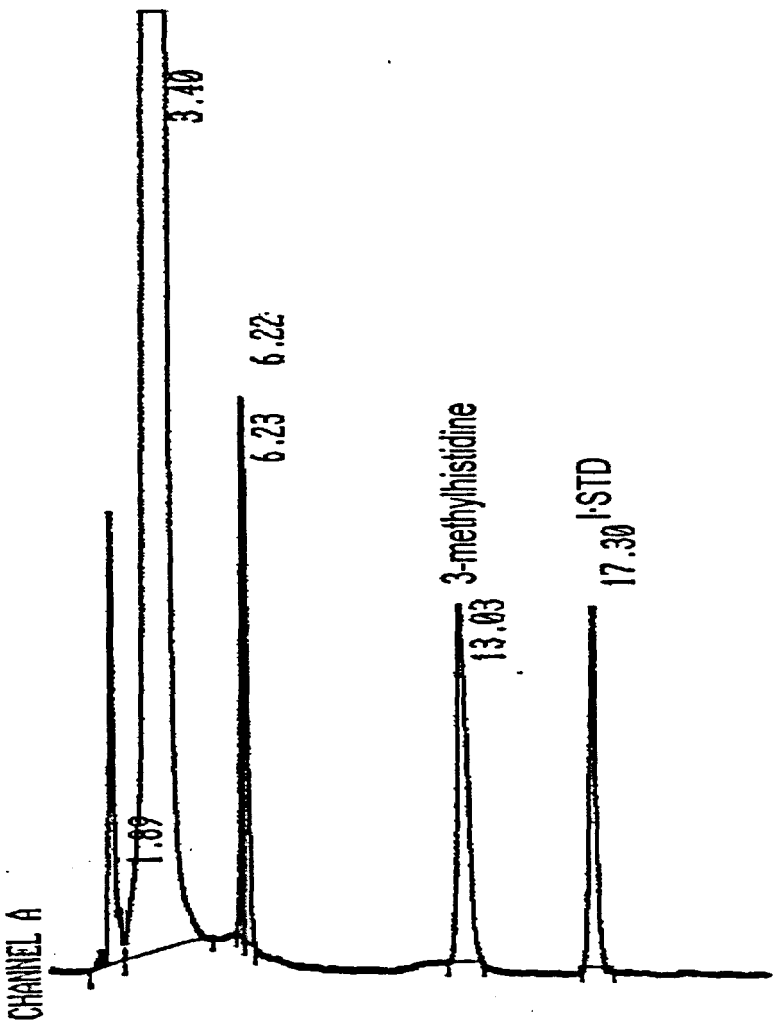


Figure 2.3 Chromatogram of a 3-methylhistidine standard for urine analysis (I·STD, internal standard).

Reagents:

1) RIA buffer, prepared by dissolving 7.8 g $NaH_2PO_4 \cdot 2H_2O$ in 900 ml distilled water and pH adjusted to 7.4 and then adding 8.77 g sodium chloride, 0.2 g sodium azide and 5 g bovine serum albumin (BSA) and making up to 1 l with distilled water.
2) First antibody, antiserum to ovine GH (AFP-CO123080), donated by NIH (National Institutes of Health, Maryland, USA). This was diluted 1: 15,000 with RIA buffer.
3) Second antibody comprised 16 % (v/w) polyethylene glycol (PEG), 16 % (v/v) RIA buffer, 0.03 % (v/v) normal rabbit serum and 0.83 % (v/v) anti-rabbit IgG precipitating serum [both donated by Scottish Antibody Production Unit (SAPU)].
4) Ovine GH (AFP-9220A, donated by NIH) stock standard (10 μg/ml), prepared by dissolving 220 μg ovine GH with 440 μl of 7.5 % sodium bicarbonate. This was diluted 1: 50 with RIA buffer and batched in 100 μl aliquots in Eppendorf tubes and stored at -20 °C.

Procedure: Standards ranging in concentration from 0.15 to 40 ng/ml were prepared by diluting the stock standard with RIA buffer. A volume of 150 μl of standard or sample was transferred to a RIA tube and 50 μl of first antibody were added, mixed and incubated at room temperature for 6 hours. One hundred microlitres of ^{125}I-GH (10,000 cpm) were added to the tubes and incubated overnight at room temperature. After the addition of second antibody (300μl), tubes were incubated at room temperature for a further 2 hours, then centrifuged at 3,000 g for 30 minutes and the supernatant decanted. The protein precipitate containing antibody-bound ^{125}I-GH was counted on a gamma counter (Cobra Auto-gamma, Packard, Berks, UK). The concentration of GH in the samples was determined by interpolation from a standard curve.

2.3.23 IGF-I 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.

Reagents:

1) RIA buffer, as described in GH RIA.
2) Extraction medium, prepared by mixing 7 parts of ethanol with 1 part of 2 M HCl.
3) Neutralizing buffer, prepared by dissolving 4 g TRIS in 100 ml RIA buffer.
4) First antibody, polyclonal rabbit anti-rhIGF-I, donated by NIDDK (Bethesda, Maryland, USA).
5) Second antibody comprised 16 % (v/w) PEG, 16 % (v/v) RIA buffer, 0.4 % (v/v) normal rabbit serum and 6 % (v/v) anti-rabbit IgG precipitating serum (SAPU).
6) Standard, recombinant human IGF-I (Bachem, Saffron Walden, Essex, UK).

Procedure: The concentration of standards ranged from 10 to 2,500 ng/ml. The extraction was achieved by adding 4 volumes of the extraction medium to 1 volume of the samples and the standard, and incubating at room temperature for 30 minutes. This solution was centrifuged at 3,000 g for 10 minutes, after which a specific amount of supernatant was removed and an equal volume of neutralizing buffer was added and then the samples were diluted with RIA buffer. The first antibody at a dilution of 1: 2,000 was added to standards and sample tubes and incubated for 24 hours before adding ^{125}IGF-I (approximately 20,000 cpm per tube) and then incubated overnight at room temperature. The second antibody 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 GH.

2.3.24 Insulin Enzyme Linked ImmunoSorbent Assay (ELISA)

The concentration of insulin in plasma was determined using an ELISA in a commercial kit (Mercodia, Uppsala, Sweden). It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to a microtitration well. A simple washing step removes unbound enzyme-labelled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

2.3.25 Mammary blood flow

Blood velocity and a cross-sectional area of milk vein were measured for calculating mammary blood flow by the method of Christensen *et al.* (1989) using Doppler ultrasound equipment (Ausonics Impact, Ausonics International Inc., CA, USA) coupled to a video cassette recorder (VCR) and an external monitor. The procedure was carried out in stalls with the cow standing still. The milk vein entering the abdominal wall was clipped and the vein marked with a marker pen. The probe coated with ultrasound scanning gel (BCF Technology Ltd., Livingston, UK) was placed at the point where the milk vein entered the abdominal wall, to keep a Doppler angle of 0 °. The velocity of the blood (m/sec) was measured by a pulsed wave Doppler mode with a Linear/Curved Linear Array transducer and 100 Hz wall filter. The processing signal of the blood velocity was recorded with the VCR. Maximal velocity (V_{max}) was measured with a trace mode and then calculated as $V_{max/2}$. The cross-sectional area (cm^2) of milk vein was

measured by placing the probe on to the surface of the vein with minimal pressure on to the surface of vein. Frequency was set at 7.5 MHz. The image of the cross-sectional area was recorded with the VCR and calculated in the elliptic mode.

2.4 MAMMARY TISSUE ANALYSIS

2.4.1 Preparation of tissue samples

Enzyme and DNA assay

Preparation of tissue samples for enzyme and DNA assay was based on the method of Wilde *et al.* (1986). Frozen mammary tissue was weighed and homogenized at 4 °C in 9 volumes of an iso-osmotic TRIS-sucrose buffer (0.03 M TRIS, 0.3 M sucrose, 1 mM reduced glutathione (GSH) and 1 mM EDTA; pH 7.4) for 15 seconds at 80 % maximum speed in a homogenizer (IKA Labortechnik, Staufen, Germany). The homogenized samples were centrifuged at 11,000 g for 60 seconds to obtain particle-free supernatant (PFS) and homogenate, which were stored at -20 °C until analysis.

Immunohistochemistry

Tissue samples were fixed overnight in 4 % formalin (Sigma, Poole, UK) and stored in 70% (v/v) ethanol until treated. Tissues were washed in absolute ethanol at room temperature for 3 hours with three changes of ethanol, followed by washing with absolute ethanol: chloroform (1:1, v/v) for 1 hour. After a further washing with chloroform for 1 hour, the tissues were incubated with paraffin in a oven at 60 °C for 2 hours with two changes of paraffin and then placed in a embedding-moulds with paraffin. Tissues were processed into 4 μm thick sections on a microtome (Reichert-Jung, 2040 Autocut, Nussloch, Germany) using disposable steel blades (Leica model 819, Nussloch, Germany). Sections were floated on to a warm water bath and mounted on to a silane-coated slides and air dried overnight.

2.4.2 Acetyl CoA carboxylase (ACC)

The activity of ACC was determined by addition of a radioactively labelled precursor to a reaction mixture and measuring its incorporation over a fixed incubation time using a modification of the method described by Ingle *et al.* (1973).

Reagents:

1) Stock solution comprised 200 mM TRIS (pH 7.5), 100 mM $MgCl_2$, 200 mM citrate (pH 7.5), 1 mM EDTA, 5 % (w/v) BSA and 100 mM GSH.
2) Assay solution, prepared by adding 50 mM ATP (pH 7.5), 4.45 mM acetyl CoA and 1.35 μCi sodium bicarbonate (specific activity 0.1 mCi/mmol, Amersham, Buckinghamshire, UK) to the stock solution.

Procedure: An aliquot (150 μl) of PFS was transferred into a screw-capped glass scintillation vial containing 20 μl distilled water and 330 μl stock solution, mixed and pre-incubated at 37 °C in a water bath for 30 minutes. The assay solution was warmed to 37 °C with acetyl CoA omitted from the blank. The reaction was initiated by addition of 0.5 ml assay solution to the pre-incubated solution, the mixture further incubated for 90 seconds and stopped by adding 0.2 ml of 5 M HCl. The mixture was heated at 55 °C on a heating module for 1 hour and then ground dry ice added and mixed to evaporate unreacted sodium bicarbonate. After a further evaporation with dry ice, 10 ml of scintillation fluid (Emulsifier Safe, Packard, Meriden, USA) were added and the mixture thoroughly shaken and counted for ^{14}C content (^{14}C 4 minute counts; Packard 1600TR liquid scintillation analyser). The ACC activity was expressed as the number of nanomoles of bicarbonate incorporated per minute per mg DNA.

2.4.3 Fatty acid synthetase (FAS)

FAS activity was determined by the method of Speake *et al.* (1975). FAS carries out elongation of fatty acid chains in the process of fat synthesis. The first reduction step catalysed by the enzyme produces $NADP^+$ whose production can be monitored spectrophotometrically, giving a direct measurement of enzyme activity.

Reagents: 0.25 M KH_2PO_4 (pH 6.6) containing 1.5 mM EDTA and 1.5 mM GSH.

Procedure: An aliquot (25 µl) of PFS, which had previously been diluted 1: 5 with iso-osmotic TRIS-sucrose buffer, was added to a 1.5 ml plastic cuvette containing 100 µl of 1 mM NADPH (Boehringer Mannheim, Lewes, UK) and 795 µl of 0.25 M KH_2PO_4, mixed and incubated at 30 °C for 5 minutes with the absorbance measured at 340 nm (Cecil CE5501 double beam UV spectrophotometer, Cambridge, UK). Fifty microlitres of 0.6 mM acetyl CoA were added to the cuvette, mixed, incubated and absorbance measured at 30 °C for 5 minutes before the addition of 30 µl of 1.3 mM malonyl CoA. Again, the solution was mixed, incubated and absorbance measured at 30 °C for 5 minute. The activity of FAS was expressed as the number of micromoles of NADPH incorporated into product per minute per mg DNA.

2.4.4 Galactosyltransferase (GT)

GT activity was determined by the method of Kuhn and White (1977). Disruption of the Golgi membranes allows free access of substrates to the enzyme complex and its activity can be determined by incorporation of a radiolabelled precursor into the end-product.

Reagents:

1) Reaction solution, prepared by mixing 0.2 M TES buffer (pH 7.4), 0.15 M $MnCl_2$ and 5 % (v/v) Triton X-100 in a ratio of 2: 2: 1.

2) 5 mM UDP-[^{14}C]-galactose (25,000dpm per 5 µl), prepared by dissolving 6.5 mg UDP-galactose in 1.82 ml distilled water and then adding 180 µl of UDP-[^{14}C]-galactose.

Procedure: Five microlitres of 5 mM UDP-[^{14}C]-galactose were transferred into a 0.5-ml microtube containing 25 µl of reaction solution and 10 µl of 0.1 M N-acetylglucosamine and mixed. The N-acetylglucosamine was omitted from the blank. The reaction was initiated by the addition of sample (10 µl homogenate), incubated in an oven at 37 °C for 10 minutes and stopped by placing tubes in a boiling water bath for 90 seconds. The tubes were placed on ice, 50 µl distilled water added and transferred to the top of a DOWEX resin column (1 ml, formate form, 200 - 400 mesh, 4 % crosslinking). The washed solution was collected in a scintillation vial. The tubes were washed twice more with distilled water (2 x 100 µl), the washings also being transferred to the column. One ml of distilled water was added on to the column to elute reaction product and 10 ml scintillation fluid added, shaken and counted for ^{14}C content. The GT activity was expressed as the number of micromoles of galactose incorporated per minute per mg DNA.

2.4.5 DNA

The concentration of DNA in mammary tissue was determined by the method of Labarca and Paigen (1980). The method is based on the enhancement of fluorescence when bisbenzimidazole (Hoechst 33258) binds to DNA.

Reagents:

1) Hoechst 33258 solution, prepared by dissolving 10 mg of Hoechst 33258 with 10 ml of distilled water.

2) TNE buffer solution (pH 7.4) comprised 100 mM TRIS, 10 mM EDTA and 2 M sodium chloride.

3) Assay solution, prepared by transferring 10 ml TNE buffer solution and 10 µl Hoechst 33258 solution into a 100-ml volumetric flask and making up to volume with distilled water.

4) DNA Standard (10 µg/ml), prepared by dissolving 10 mg of calf thymus DNA in 10 ml of 10 % (v/v) TNE buffer solution. This was further diluted 1: 100 with 10 % (v/v) TNE buffer solution.

Procedure: The homogenate samples were diluted 1: 10 with 10 % TNE buffer stock solution. The fluoresence was measured using DyNA Quant 200 fluorimeter (Hoefer Pharmacia Biotech Inc., CA, USA) with wavelength of 365 ± 7 nm excitation filter and a 460 ± 15 nm emission filter.

2.4.6 Proliferating cell nuclear antigen (PCNA)

PCNA was analysed by immunohistochemistry using the Streptavidin-biotin staining procedure as described by DAKO (Cambridge, UK).

Reagents:

1) Phosphate buffered saline (PBS) comprised 0.1 M sodium chloride, 9 mM Na_2HPO_4 and 1.3 mM NaH_2PO_4.

2) 2 % (v/v) hydrogen peroxide solution, prepared by mixing 10 ml hydrogen peroxide with 140 ml methanol.

3) 0.5 % (w/v) BSA solution, prepared by dissolving 0.5 g BSA in 10 ml PBS.

Procedure: Paraffin-embedded sections were dewaxed and rehydrated by washing with the following solutions in sequence: histoclear (5 min x 2), absolute ethanol (10 min), 90 % ethanol (3 min), 70 % ethanol (3 min). After a further washing with PBS for 5 minutes,

endogenous peroxidase activity was blocked with 2 % hydrogen peroxide solution for 5 minutes at room temperature followed by two washes with PBS (5 min x 2). The primary mouse anti-PCNA (DAKO) was diluted 1: 50 with 0.5 % BSA solution and 0.1 ml was applied to the slide, which was then incubated at room temperature for 1 hour in a humidified chamber. Unbound antibodies were removed by washing the sections with PBS (5 min x 2). They were then incubated with biotinylated rabbit anti-mouse IgG (DAKO), which had previously been diluted 1: 1000 with 0.5 % BSA solution, at room temperature for 1 hour in a humidified chamber. The avidin-biotin complex (DAKO) was prepared according to manufacturer's instructions and applied to sections for 45 minutes in a humidified chamber followed by washing with PBS. The bound antibodies were stained by incubating the sections with 3,3'-diaminobenzidine (DAB, Sigma) solution prepared as instructed by the manufacturer. After the staining was monitored under the microscope the sections were washed in distilled water. The sections were then placed in Mayers hematoxylin (Sigma) for 5 minutes and rinsed with water. To develop the dye, the sections were placed in 10 % of Scott's tap water (Sigma) until all the cell nuclei stained blue. They were washed with distilled water and dehydrated through ascending ethanols and cleared with histoclear and mounted with DPX mounting medium (Agar Scientific, Stansted, UK).

Cells which stained positive for PCNA were quantified by using an image analysis system (Leica Q500MC, Nussloch, Germany) where, at least, 1000 nuclei per slide were counted.

CHAPTER THREE

EFFECTS OF CHANGES IN DIETARY AMINO ACID BALANCE ON MILK YIELD AND MAMMARY FUNCTION IN DAIRY COWS

3.1 INTRODUCTION

Both fish meal and feather meal have a similar, high content of rumen-undegradable protein (UDP) but differ widely in amino acid composition. In contrast to fish meal, feather meal is known to contain low concentrations of methionine, lysine and histidine (Chamberlain *et al.*, 1992). Compared with fish meal, when feather meal was given as the sole protein supplement to dairy cows consuming grass silage, it proved to be of low nutritional value, indicating the importance of the amino acid composition of protein supplements in determining their nutritional value for the dairy cow. This is further supported by the findings that intravenous infusions of four AAs, methionine, lysine, histidine and tryptophan, led to sizeable increases of milk production in cows consuming a diet of grass silage and a cereal-based supplement containing feather meal (Choung and Chamberlain, 1995). In later experiments under similar dietary conditions, histidine was shown to be first-limiting for milk production (Kim *et al.*, 1999; 2001a).

With respect to the low efficiencies of transfer of amino acids to milk protein, it has been pointed out that low values for histidine might reflect its use for replenishing body protein, especially in short-term experiments (Kim *et al.*, 2001a). Furthermore, in severe undernutrition, cows can mobilize up to 300 g of protein a day to support milk production (Botts *et al.*, 1979) and this suggests that the mobilization of body protein might mask the effects of dietary deficiencies of amino acids in the short term.

Although frequent milking can lead to an increase of milk production and changes of mammary function such as increases of key enzyme activities and cell numbers in the

mammary gland of goats (Henderson *et al.*, 1985; Wilde *et al.*, 1987a), little is known about the effects of nutrition, particularly deficiencies in the supply of specific amino acids, on mammary function in the dairy cow. The objective of the present experiments was to examine the longer-term effects of amino acid balance on milk production and mammary function in cows receiving grass silage.

Although it was recognized that feather meal would not normally be given to dairy cows as the sole protein supplement, the objective here was to ascertain the importance of amino acid balance on milk production. Fish meal and feather meal were chosen as two extremes of amino acid balance, which would be expected (Chamberlain *et al.*, 1992) to result in sizeable differences in milk production.

3.2 EXPERIMENTAL

3.2.1 Animals and their management

Twelve Friesian cows in their first to fifth lactations were used in Experiments 1 and 2. They were 3 – 8 and 15 – 24 weeks into their lactation at the start of Experiments 1 and 2, respectively. Average body weight of the cows was 571 kg (range 504 – 696 kg) in Experiment 1 and 621 kg (range 545 – 760 kg) in Experiment 2. The animals were housed individually in metabolism stalls with water freely accessible and milked each day at 07.00 and 15.00 h. Food was provided in two equal meals, one after each milking.

In both experiments cows were given a basal diet consisting of *ad libitum* access to grass silage and 3 kg/d of sugar beet pulp. The amount of silage offered was adjusted to ensure a daily refusal of around 15 % of that offered. For all experiments, the silage was made from perennial ryegrass (*Lolium perenne*) cut at an early stage of growth and ensiled with the addition of an inoculum of *Lactobacillus plantarum* (Ecosyl; ICL pcl, Billingham, UK) at 3 litres/tonne in a bunker silo of 70-tonne capacity. The chemical

compositions of the silage and the supplements are shown in Table 3.1 and 3.2, respectively.

3.2.2 Experimental treatments and design

3.2.2.1 Experiment 1

Experiment 1 was carried out between lactation weeks 5 and 16. The experiment consisted of three periods. Cows were given a control diet consisting of the basal diet as described above plus 5 kg/d of fish meal (a pelleted mixture of 50 % rolled barley, 30 % fish meal and 20 % citrus pulp on a fresh weight basis) for 2 weeks in period 1 (lactation weeks 5 and 6) and split, on the basis of parity and milk yield, into two groups at the end of period 1. The control group continued on the control diet for the whole experiment. For the treatment group, the fish meal was replaced by an equivalent amount of UDP as 5 kg/d of feather meal (a pelleted mixture of 50 % rolled barley, 25 % feather meal and 25 % citrus pulp on a fresh weight basis) for 6 weeks in period 2 (lactation weeks between 7 and 12) after which they received the control diet for 4 weeks in period 3 (lactation weeks between 13 and 16).

Food intake and milk yield from each half-udder (two diagonally opposed quarters) were recorded daily. The composition of milk was determined on a representative, composite sample from four consecutive milkings just before the last day of each period. Samples of blood were taken from a tail vessel at 09:30 and 14:00 on the day before the end of each period. Additional samples of blood and milk were taken on the last day of the second week in period 2 and 3. Measurements of empty udder volume were made before the mammary tissue biopsy on the last day of each period.

Table 3.1 The chemical composition (g/kg DM, unless stated otherwise) of the silages used in the experiments

	Experiment 1	Experiment 2
DM (g/kg)	238	221
Organic matter	919	924
Total N	24.2	20.9
NPN (g/kg N)	739	719
Ammonia-N (g/kg N)	96	116
Water-soluble carbohydrate	38	17
Neutral-detergent fibre	562	588
Acid-detergent fibre	356	360
pH	3.9	3.8
Lactic acid	125	114
Acetic acid	17	12
Butyric acid	2	2
Ethanol	21	29
DOMD[1]	631	663

[1]Digestible organic matter in the dry matter.

Table 3.2 The chemical composition (g/kg DM, unless stated otherwise) of the fish meal cube, feather meal cube and sugar beet pulp used in the experiments

	Fish meal cube	**Feather meal cube**	**Sugar beet pulp**
DM (g/kg)	869	884	872
Organic matter	878	917	882
Total N	47.3	48.2	17.9
Starch	277	266	5
Sugars	61	76	219
Neutral-detergent fibre	269	265	350
Acid-detergent fibre	69	86	200

3.2.2.2 Experiment 2

Cows were given a break period of 5 weeks after completion of Experiment 1. During the break period, cows were given access to a bare grass field for a restricted time (around 6 h) during the day and each group was housed separately in metabolism cubicles for feeding a mixture of silage, fish meal as described above and sugar beet pulp (0.89: 0.03: 0.08 on a fresh weight basis). Experiment 2 repeated the procedure used in Experiment 1 between lactation weeks 22 and 32 in the same cows, with the exception that period 1 consisted of 1 week instead of 2 weeks.

Recording of food intake and milk yield and composition were as described for Experiment 1. As in Experiment 1, samples of blood and mammary tissue were taken, and the volume of empty udder was determined.

3.2.3 Chemical analysis

Minced wet silage was analysed for DM by toluene distillation, total nitrogen, true protein, ammonia, lactic acid, ethanol, water-soluble carbohydrate, VFA and pH. Dried samples of silage were analysed for ash, NDF, ADF and digestible organic matter in the dry matter (DOMD). Concentrate samples were analysed for DM, total nitrogen, ash, NDF, ADF, sugars and starch. Blood plasma samples were analysed for albumin, non-esterified fatty acids, glucose, urea and free AAs, and whole blood samples for total haemoglobin. Milk samples were analysed for fat, crude protein, and lactose. Mammary tissue samples were analysed for DNA, acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS) and galactosyltransferase (GT). Fatty acid composition of milk fat and proliferating cell nuclear antigen (PCNA) in mammary tissue were analysed in Experiment 1.

3.2.4 Statistical analysis

For statistical analysis, mean values for feed intake and milk yield were taken for the last 7 d of each experimental period. One of the cows became lame during the period 1 in Experiment 1 and was replaced before the start of period 2. In Experiment 1, one cow between lactation weeks 10 and 12, and another cow in lactation week 14 were unwell, and their milk yield and silage intake decreased. Although they recovered well during the rest of the experiment, the results for these animals for those lactation weeks were omitted from the statistical analysis. In Experiment 2, one cow became unwell between lactation weeks 23 and 26, and her silage intake and milk yield decreased. She recovered well during the rest of the experiment, but the results for this animal for those lactation weeks were omitted from the statistical analysis. Treatment differences were compared for period 2 and 3 separately by analysis of covariance with the period 1 response as a covariate, using the directives of Genstat 5 (Lawes Agricultural Trust, 1990). The statistical model used was:

$$Y = \text{Overall mean} + \text{Treatment} + b(\text{covariate} - \text{covariate mean}) + \text{Error}$$

where Y is response

b is covariate coefficient

3.3 RESULTS

3.3.1 Experiment 1

To present an overview of the results from the two experiments, milk yields from both Experiments 1 and 2 are summarised in Figure 3.1. Milk yields from the half-udder, from which the mammary biopsy was taken, are not presented since milk yield was markedly decreased and recovery took approximately 2 weeks after mammary tissue biopsy. The mean rate of decline in milk yield in the control group was 1.9 % and 1.6 %

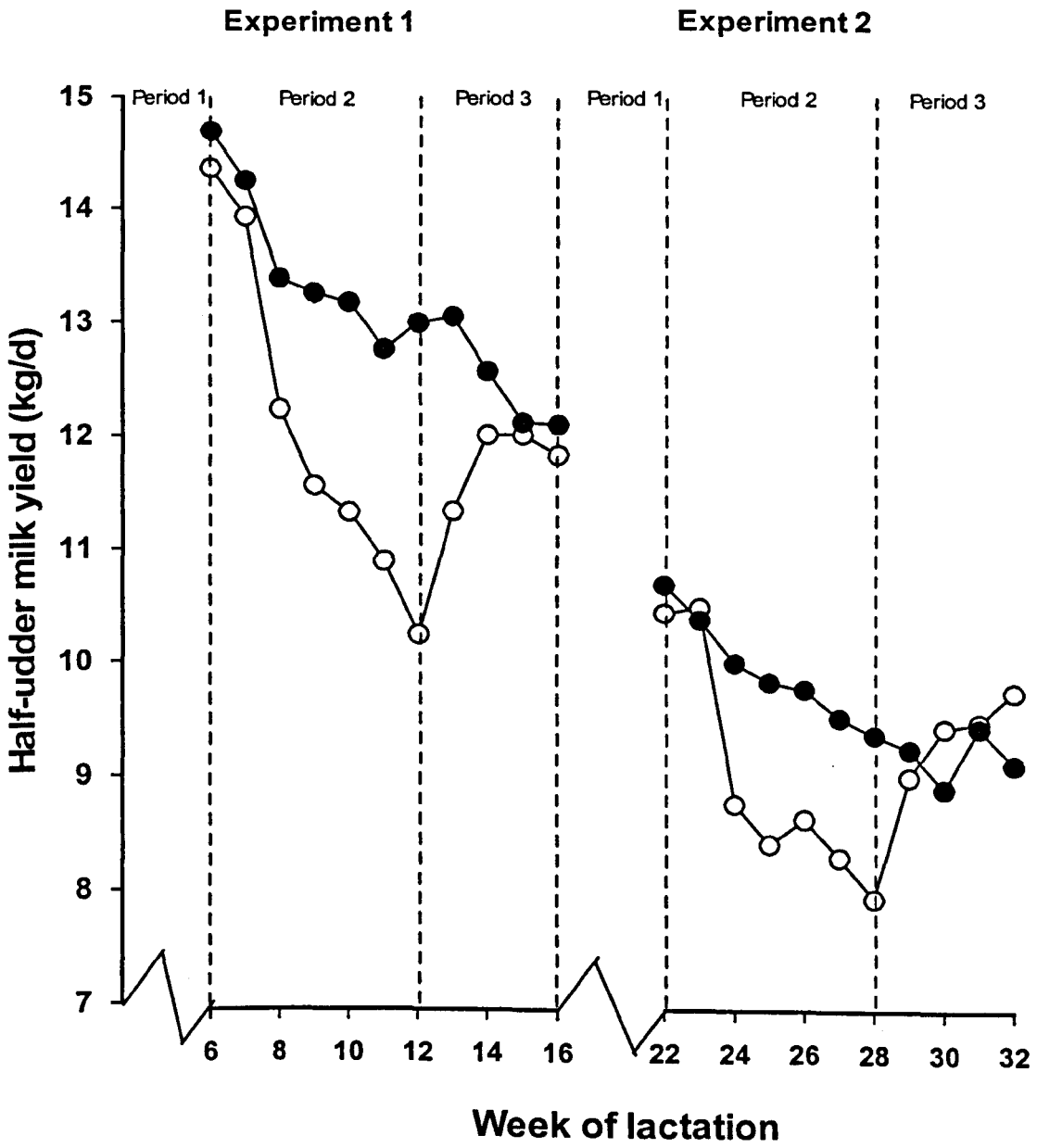


Figure 3.1 Half-udder milk yield (kg/d) in Experiments 1 and 2. The control group (●) received fish meal throughout the experiments; the treatment group (O) received fish meal until week 6 of lactation when they were changed to feather meal before returning to fish meal for 4 weeks (Experiment 1). After a break of 5 weeks, the experimental procedure was repeated using the same cows (Experiment 2).

per week in Experiments 1 and 2, respectively. During period 1 in Experiments 1 and 2 when both groups were given the control diet, there were no significant differences in milk yield between control and treatment groups. However in period 2, when the treatment group was transferred to the feather meal diet, the milk yield of the treatment group was progressively decreased from the second week of period 2 until the end of period 2 compared with that of the control group, and this was true also in Experiment 2. The fall of milk yield in the treatment group was completely recovered by a return to the control diet for 4 weeks in period 3 in both experiments.

Results for feed intake, milk production and body weight in Experiment 1 are shown in Table 3.3. After the fish meal diet was replaced by the feather meal diet, the silage intake of the treatment group was significantly ($P<0.05$) decreased during period 2. Yields of both protein and lactose in the treatment group were reduced at the end of period 2 compared with those in the control group ($P<0.01$ and $P<0.05$, respectively), but the yield of protein in the treatment group had also been decreased ($P<0.05$) in the second week of period 2. Changing the protein supplement in the diet did not affect milk fat yield. The concentration of milk protein in the treatment group tended to be decreased ($P<0.10$) and that of milk fat was significantly ($P<0.05$) increased at the end of period 2. After the treatment group was transferred to the control diet, within one week, there were no significant differences in feed intake and milk production between treatments in period 3.

The concentrations and yields of $C_{10:0}$ and $C_{12:0}$ fatty acids in the treatment group were significantly ($P<0.05$) decreased at the end of period 2, but the concentration of the sum of $C_{16:0}$ and $C_{16:1}$ fatty acids was significantly ($P<0.05$) higher than that in the control group (Table 3.4 and 3.5). No significant differences in fatty acid composition of milk fat between treatments were evident in period 3.

Table 3.3 Feed intake, milk production and body weight in Experiment 1

	Period 1 (wk[4] 6)			Period 2 (wk 8)			Period 2 (wk 12)			Period 3 (wk 14)			Period 3 (wk 16)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
DM intake, kg/d															
Silage	12.3	12.0	0.78	12.0	10.4	0.59*	12.8	11.2	0.48*	12.6	12.1	0.58	12.7	12.6	0.40
Total	19.2	19.1	0.78	19.0	17.4	0.59*	19.8	18.1	0.48*	19.6	19.1	0.57	19.7	19.6	0.40
Milk yield, kg/d	14.7	14.4	0.88	13.4	12.2	1.06	13.0	10.3	0.94*	12.6	12.0	0.76	12.1	11.8	1.02
Milk protein, g/kg	30.8	29.7	0.83	31.0	28.9	1.21	32.2	29.7	1.29	32.0	31.8	1.06	32.8	33.2	1.19
g/d	451	424	19.2	412	351	24.7*	415	304	22.4**	399	381	21.9	395	392	27.9
Milk fat, g/kg	35.6	39.2	2.71	39.7	39.8	2.27	37.0	43.1	1.98*	38.4	40.2	2.69	36.7	40.6	2.01
g/d	522	563	52.3	531	487	40.7	468	448	24.4	483	476	37.9	455	469	51.4
Milk lactose, g/kg	48.7	49.4	0.76	47.8	49.3	0.97	47.4	46.6	1.02	47.9	47.4	0.71	46.7	46.0	1.13
g/d	715	709	42.5	638	602	53.5	616	479	48.8*	605	563	39.2	569	540	53.6
Body weight, kg	607	575	29.7	593	590	10.4	605	610	15.9	609	616	16.9	607	617	17.4

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
* P<0.05, ** P<0.01.

Table 3.4 The fatty acid composition (g/kg) of milk fat in Experiment 1

	Period 1 (wk[4] 6)			Period 2 (wk 8)			Period 2 (wk 12)			Period 3 (wk 14)			Period 3 (wk 16)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
$C_{4:0}$	79	78	5.7	79	87	5.3	74	76	5.8	81	82	2.8	66	171	63.0
$C_{6:0}$	32	33	1.9	31	32	1.7	33	30	1.8	33	32	1.0	32	36	2.1
$C_{8:0}$	17	17	1.3	16	16	0.9	17	15	1.0	17	18	0.7	17	19	2.3
$C_{10:0}$	36	37	4.1	35	34	1.6	39	32	2.3*	39	40	2.2	41	37	5.4
$C_{12:0}$	44	44	5.7	37	38	1.9	45	37	2.7*	44	45	2.3	50	45	6.4
$C_{14:0}$	119	116	10.5	112	118	3.7	131	95	20.0	131	132	5.2	141	130	13.8
$C_{4:0}$ - $C_{14:0}$	327	325	24.9	309	325	11.2	337	285	22.8	345	349	11.2	345	439	42.6
$C_{16:0}$	323	299	22.9	316	337	13.2	348	402	24.1	342	338	7.8	372	319	28.9
$C_{16:1}$	24	24	2.1	30	21	6.4	22	23	1.8	24	20	2.3	25	20	2.8
$C_{16:0}$ - $C_{16:1}$	347	323	22.5	347	357	19.5	369	427	23.4*	365	359	7.8	397	339	30.4
$C_{18:0}$	98	103	10.1	96	96	5.4	91	99	10.3	85	92	5.8	75	72	7.9
$C_{18:1}$	218	227	29.3	223	199	16.0	174	188	15.4	177	174	8.6	164	145	10.2
$C_{18:2}$	5	11	3.9	11	18	2.3*	10	7	3.4	16	21	4.5	8	6	3.9
$C_{18:3}$	5	11	3.5	10	9	1.0	7	7	3.6	10	7	1.9	7	4	2.8
$C_{18:0}$ - $C_{18:3}$	326	352	39.4	344	319	18.2	289	294	25.4	290	292	13.1	255	225	20.4

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
* $P<0.05$.

Table 3.5 The yield of fatty acids (g/d) in milk fat in Experiment 1

	Period 1 (wk[4] 6)			Period 2 (wk 8)			Period 2 (wk 12)			Period 3 (wk 14)			Period 3 (wk 16)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
$C_{4:0}$	42	44	5.9	43	41	5.3	35	33	3.0	39	39	4.2	29	79	30.2
$C_{6:0}$	17	19	2.5	16	16	1.6	15	13	1.2	16	16	1.5	14	17	2.6
$C_{8:0}$	9	10	1.4	8	8	0.7	8	7	0.7	8	9	0.8	8	9	1.2
$C_{10:0}$	19	21	3.4	18	16	1.5	19	14	1.5*	19	19	1.5	18	18	3.1
$C_{12:0}$	24	25	4.7	20	18	1.7	21	16	1.8*	21	22	1.7	23	21	3.3
$C_{14:0}$	63	66	10.4	61	55	4.4	62	43	9.7	63	63	5.7	65	61	9.4
$C_{4:0}$ - $C_{14:0}$	173	183	26.8	166	154	12.9	160	127	15.3	166	167	14.7	157	204	25.9
$C_{16:0}$	170	167	20.9	174	155	8.9	169	172	11.6	165	161	13.2	173	147	19.8
$C_{16:1}$	12	13	1.4	17	10	4.9	10	10	0.7	12	10	1.2	11	9	1.2
$C_{16:0}$ - $C_{16:1}$	183	181	21.6	191	165	9.1*	180	182	12.1	177	170	13.2	184	156	20.4
$C_{18:0}$	51	58	7.0	54	45	7.8	44	43	7.3	42	44	5.5	35	33	6.7
$C_{18:1}$	110	129	15.1	128	91	21.5	85	82	11.9	88	80	5.7	77	66	10.3
$C_{18:2}$	3	6	2.4	6	9	1.5	5	3	1.7	8	10	2.2	4	2	1.9
$C_{18:3}$	3	6	1.9	6	4	1.0	3	3	1.9	5	3	0.8	3	2	1.5
$C_{18:0}$ - $C_{18:3}$	166	199	23.0	194	148	31.1	140	128	20.6	145	135	10.8	120	102	17.4

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
*P<0.05.

The concentrations of blood plasma metabolites are given in Table 3.6. The concentration of plasma glucose in the treatment group tended to be higher ($P<0.10$) than that in the control group at the end of period 2 and remained higher until the second week of period 3. Although there were no significant differences in NEFA between treatments in any period, the concentrations of NEFA in both treatments were numerically higher in period 1 than in the other periods. There was a significant difference in urea level between treatments in period 1.

The concentrations of plasma free amino acids are shown in Table 3.7. Compared with the fish meal diet, the feather meal diet increased total essential amino acids (EAA), non-essential amino acids (NEAA) and total amino acids (TAA) in lactation week 8 but the differences were not evident in the other periods. The concentrations of His and Met were significantly lower ($P<0.01$ and $P<0.05$, respectively) in the treatment group than in the control group in lactation week 8, and lower concentrations of Arg, Met, Lys, Glu and Asn in the treatment group were seen at the end of period 2 compared with those in the control group. Increases in the concentrations of Thr, Ser, Gly, Ala, Pro and branched-chain amino acids, Val, Ile and Leu in the treatment group were evident in lactation week 8 but were only evident for Ser and Pro at the end of period 2. There were no significant differences in the concentration of plasma amino acids between treatments in period 3, with the exception that lower concentrations of His and higher concentrations of Gly in the treatment group were found in lactation week 14.

Except that fatty acid synthetase and mammary cell proliferation were lower ($P<0.05$) in the treatment group than in the control group in period 1, no significant differences were found in mammary enzyme activities, mammary cell proliferation (PCNA), total DNA or udder volume between treatments in all periods (Table 3.8).

Table 3.6 The composition of plasma and blood in Experiment 1

	Period 1 (wk[4] 6)			Period 2 (wk 8)			Period 2 (wk 12)			Period 3 (wk 14)			Period 3 (wk 16)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
Glucose, mmol/l	4.08	3.77	0.287	3.89	3.96	0.151	3.52	3.80	0.131	3.59	3.85	0.125	3.63	3.83	0.148
Urea – N, mmol/l	4.48	5.29	0.265*	5.28	5.65	0.579	5.73	5.30	0.511	5.73	5.11	0.471	5.49	5.16	0.280
Haemoglobin, g/l	116	109	4.3	106	105	5.8	88	87	5.6	84	83	7.5	92	92	6.6
Albumin, g/l	40	39	0.9	39	38	1.6	39	41	1.8	36	39	1.6	37	40	1.6
NEFA[5], mmol/l	0.19	0.13	0.056	0.07	0.06	0.013	0.04	0.04	0.017	0.02	0.05	0.012	0.04	0.04	0.008

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
[5] Non-esterified fatty acids.
* $P<0.05$.

Table 3.7 The concentrations (μmol/l) of plasma free amino acids in Experiment 1

	Period 1 (wk[4] 6)			Period 2 (wk 8)			Period 2 (wk 12)			Period 3 (wk 14)			Period 3 (wk 16)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
Histidine	19	19	3.6	19	10	2.4**	15	15	2.6	30	17	5.2*	15	16	2.4
Threonine	126	119	11.9	115	149	12.1*	119	90	12.5	100	108	15.7	125	124	12.7
Arginine	66	68	5.3	70	69	9.1	80	52	7.3**	76	63	10.8	86	81	14.1
Tryptophan	37	38	2.2	45	47	4.9	35	28	3.0	31	33	4.5	41	40	3.3
Methionine	22	24	2.7	26	16	2.9*	24	10	1.3***	22	21	2.4	26	25	2.4
Valine	189	209	17.4	195	397	35.9***	202	269	35.3	194	161	32.4	196	203	31.9
Phenylalanine	40	46	2.6	51	59	4.8	41	46	4.6	45	40	3.3	41	44	2.6
Isoleucine	119	141	18.6	109	153	17.1*	106	96	17.8	95	91	14.5	107	114	19.6
Leucine	100	103	9.3	97	141	12.7**	89	95	12.0	89	71	8.7	91	98	10.9
Lysine	69	72	4.1	76	64	9.0	69	36	10.8*	67	53	8.0	75	74	11.3
Aspartic acid	11	9	1.0	0	2	2.8	0	0	0	5	3	1.3	0	0	0
Glutamic acid	71	74	3.1	74	71	7.6	55	42	4.3*	60	49	5.7	53	53	4.4
Serine	101	104	12.8	73	135	12.1***	75	105	11.1*	89	97	13.1	90	99	6.8
Glycine	458	437	62.6	427	622	54.0**	442	474	71.3	340	405	19.1*	367	405	42.5
Alanine	193	160	24.9	163	194	13.1*	166	142	17.1	178	194	15.1	174	202	13.9
Tyrosine	51	58	5.6	88	110	12.4	77	76	4.8	49	42	5.8	65	67	4.6
Asparagine	44	46	1.9	47	53	4.6	50	32	4.2**	47	42	3.9	52	54	4.1
Taurine	44	35	2.9*	50	45	6.5	46	34	10.6	32	33	6.1	42	34	14.0
Glutamine	300	337	61.1	268	283	20.2	257	243	45.8	327	319	51.2	305	294	18.0
Ornithine	37	33	2.9	32	35	5.3	30	27	4.8	29	32	5.6	34	35	4.7
Proline	56	49	4.5	44	92	8.0***	45	68	7.7*	51	56	3.9	59	73	9.2
Essential AA	786	839	50.0	790	1116	94.5**	775	740	80.7	742	666	71.7	799	822	96.6
Non-essential AA	1365	1343	77.8	1269	1638	78.2**	1252	1231	135.4	1205	1274	96.0	1249	1306	94.0
Total AA	2152	2182	105.5	2079	2734	128.8***	2029	1971	176.4	1954	1933	146.1	2051	2125	161.7

[1] Control group. [2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week. * P<0.05, ** P<0.01, ***P<0.001.

Table 3.8 Mammary enzyme activities, PCNA, total DNA and udder volume in Experiment 1

	Period 1 (wk[4] 6)			Period 2 (wk 12)			Period 3 (wk 16)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED
Acetyl-CoA carboxylase, nmol/min per mg DNA	180	131	47.0	128	217	70.2	90	81	20.2
Fatty acid synthetase, μmol/min per mg DNA	0.85	0.41	0.167*	0.67	0.38	0.150	0.49	0.26	0.118
Galactosyltransferase, μmol/min per mg DNA	0.37	0.34	0.057	0.29	0.25	0.051	0.25	0.22	0.048
PCNA[5], %	3.07	1.82	0.523*	0.41	0.79	0.285	1.84	1.35	1.359
Total DNA, g	30.6	27.8	7.86	33.3	33.5	7.20	30.5	27.0	3.44
Udder volume, l	12.3	12.1	1.96	13.0	12.9	1.27	13.7	11.8	1.08

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
[5] PCNA, proliferating cell nuclear antigen.
* $P<0.05$.

3.3.2 Experiment 2

Results for feed intake, milk production and body weight in Experiment 2 are shown in Table 3.9. Silage intake in the treatment group was significantly ($P<0.05$) decreased in lactation week 24 compared with that in the control group but there were no significant differences in silage intake between treatments in the other periods. Yields of protein and lactose in the treatment group were progressively decreased during period 2 compared with those in the control group. The concentration of milk protein in the treatment group tended to be decreased ($P<0.10$) in lactation week 24. Although there was a tendency for the concentration of milk fat to increase with the feather meal diet at the end of period 2, no significant differences in milk fat yield between treatments were found throughout the experiment. Body weight was lower for the treatment group than for the control group during period 2. After the treatment group was transferred to the control diet, within one week, there were no significant differences in feed intake and milk production between treatments in period 3.

The concentrations of blood plasma metabolites are given in Table 3.10. Plasma glucose in the treatment group remained higher ($P<0.05$) than that in the control group from lactation week 24 to week 30.

The concentrations of plasma free amino acids are shown in Table 3.11. In lactation week 24, compared with the fish meal diet, the feather meal diet increased total essential amino acids (EAA) but the differences were not evident in the other periods. Concentrations of His, Arg, Trp, Met, Lys, Asn and Gln were significantly lower in the treatment group than in the control group in lactation week 24 but only Met and Lys remained lower by the end of period 2. The feather meal diet increased the concentrations of Val, Phe, Leu and Pro in lactation week 24 and those of Val, Phe, Ser, Tyr and Pro at

Table 3.9 Feed intake, milk production and body weight in Experiment 2

	Period 1 (wk[4] 22)			Period 2 (wk 24)			Period 2 (wk 28)			Period 3 (wk 30)			Period 3 (wk 32)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
DM intake, kg/d															
Silage	12.0	11.5	0.69	12.2	11.2	0.40*	10.1	9.4	0.43	10.7	10.7	0.55	10.8	10.4	0.25
Total	18.9	18.5	0.69	19.2	18.2	0.39*	17.1	16.4	0.43	17.7	17.7	0.55	17.7	17.4	0.25
Milk yield, kg/d	10.7	10.4	0.87	10.0	8.8	0.49*	9.4	7.9	0.44*	8.9	9.4	0.41	9.1	9.8	0.39
Milk protein, g/kg	35.8	34.1	1.44	35.9	34.4	0.70	35.6	34.3	0.90	37.6	38.3	0.87	37.1	36.2	0.86
g/d	381	355	25.0	359	300	17.5*	333	273	17.8**	332	364	18.8	335	354	18.0
Milk fat, g/kg	40.6	37.5	2.47	45.4	48.6	3.78	40.1	46.3	2.95	40.2	40.6	3.40	40.1	39.5	2.98
g/d	432	387	21.2	452	429	57.4	372	369	32.9	357	378	40.6	365	382	40.9
Milk lactose, g/kg	46.6	46.7	0.83	47.6	47.8	0.49	46.5	46.7	0.69	46.7	45.9	0.42	46.4	46.2	0.54
g/d	498	487	40.7	476	419	25.5	436	372	25.1*	416	433	22.5	423	451	19.9
Body weight, kg	617	587	34.2	630	609	4.9**	618	598	7.5*	616	607	8.3	594	582	11.9

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
* P<0.05, ** P<0.01.

Table 3.10 The composition of plasma and blood in Experiment 2

	Period 1 (wk[4] 22)			Period 2 (wk 24)			Period 2 (wk 28)			Period 3 (wk 30)			Period 3 (wk 32)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
Glucose, mmol/l	3.73	3.69	0.111	3.66	4.09	0.140*	3.64	3.99	0.117*	3.57	3.81	0.096*	3.56	3.73	0.111
Urea – N, mmol/l	4.86	4.88	0.249	4.74	4.78	0.345	5.17	4.46	0.369	5.29	5.50	0.254	5.91	5.56	0.446
Haemoglobin, g/l	98	91	5.7	93	95	2.7	86	87	3.7	96	98	4.1	102	107	5.0
Albumin, g/l	39	38	1.3	38	39	1.1	35	37	1.8	39	38	1.2	38	38	1.4
NEFA[5], mmol/l	0.03	0.04	0.005	0.03	0.03	0.010	0.02	0.01	0.008	0.02	0.02	0.008	0.02	0.01	0.007

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
[5] Non-esterified fatty acids.
* $P<0.05$.

Table 3.11 The concentrations (μmol/l) of plasma free amino acids in Experiment 2

	Period 1 (wk[4] 22)			Period 2 (wk 24)			Period 2 (wk 28)			Period 3 (wk 30)			Period 3 (wk 32)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED	C	T	SED	C	T	SED
Histidine	24	21	6.9	24	10	3.0**	18	14	5.0	20	21	3.6	19	26	4.0
Threonine	121	107	19.7	117	109	13.6	112	113	17.8	111	110	8.7	118	116	12.1
Arginine	80	88	10.8	94	66	9.0*	86	74	6.8	88	87	10.3	79	81	7.3
Tryptophan	36	35	3.1	39	33	2.3*	44	38	5.9	40	42	3.4	34	37	2.6
Methionine	24	24	1.7	27	11	2.0***	22	15	3.4*	28	25	2.2	28	25	2.4
Valine	194	208	32.7	192	344	20.1***	162	260	24.4**	171	181	18.6	164	185	14.1
Phenylalanine	36	41	2.6	41	51	2.8**	37	48	2.7**	38	40	3.5	37	39	2.7
Isoleucine	111	123	18.5	119	121	7.5	94	102	11.4	107	106	9.1	97	107	6.6
Leucine	86	96	11.1	93	118	7.1**	84	102	9.0	89	92	11.9	81	87	7.1
Lysine	75	84	11.5	79	40	6.7***	71	45	7.5**	79	77	11.3	63	71	6.6
Aspartic acid	0	0	0	2.4	1.1	1.0	3.1	3.4	0.3	3.2	3.2	0.3	3.8	3.7	1.0
Glutamic acid	51	57	5.1	52	52	5.3	42	43	2.7	49	50	2.6	46	49	2.2
Serine	95	103	10.5	99	125	18.6	83	118	11.9*	75	68	4.9	79	73	6.5
Glycine	290	281	22.2	149	147	15.1	307	382	35.4	269	253	23.8	243	234	22.1
Alanine	247	305	29.5	177	159	25.3	135	130	17.0	144	147	14.1	160	162	22.6
Tyrosine	58	63	3.9	42	55	10.9	65	86	8.8*	58	52	3.3	44	42	2.4
Asparagine	49	53	4.8	52	36	4.0**	41	37	5.0	46	45	2.8	43	43	3.3
Taurine	27	23	2.8	30	22	9.5	40	39	2.7	47	51	4.6	31	28	2.8
Glutamine	267	260	29.1	278	199	29.1*	216	202	13.6	237	227	14.4	235	218	15.5
Ornithine	37	39	7.4	38	31	3.1	36	38	3.8	39	40	5.5	31	34	3.4
Proline	89	56	13.4*	56	105	16.7*	52	95	16.5*	49	51	7.3	41	51	6.3
Essential AA	786	825	80.3	825	903	31.5*	730	811	69.5	768	783	70.4	718	775	47.4
Non-essential AA	1209	1240	61.5	990	916	108.2	1034	1159	85.9	1026	975	49.7	969	925	57.2
Total AA	1995	2065	118.1	1819	1815	116.0	1769	1965	147.7	1791	1760	106.8	1688	1699	91.9

[1] Control group. [2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week. *P<0.05, ** P<0.01, ***P<0.001.

the end of period 2. There were no significant differences in the concentrations of plasma amino acids between treatments in period 3.

Galactosyltransferase (GT) was lower ($P<0.05$) and fatty acid synthetase (FAS) tended to be lower ($P<0.10$) in the treatment group than those in the control group in period 2 (Table 3.12). No significant differences were found in acetyl CoA carboxylase, total DNA or udder volume between treatments in all periods.

3.4 DISCUSSION

As expected from earlier experiments with these diets, the results from both experiments showed that the effects of changes in dietary amino acid balance on milk production were sizeable. Although there was a fall in milk yield as lactation advanced, the differences in milk yield between the two protein supplements in the two stages of lactation remained similar (21 % vs 16 % in Experiment 1 and 2, respectively). The mean differences in the yields of milk and milk protein between the two protein supplements in Experiment 1 and 2 were 19 and 23 %, respectively, which largely agree with those obtained in the previous study where corresponding figures were 18 and 23 %, respectively (Chamberlain *et al.*, 1992).

Although the feather meal diet decreased silage intake relative to the fish meal diet, it was estimated, assuming a ME concentration in the silage of 10.1 (DOMD x 0.16) MJ/kg DM and an efficiency of utilization of ME for lactation of 0.62 (Agricultural Research Council, 1980), that, in Experiment 1, the cows consumed ME in excess of their requirements for maintenance and milk production by approximately 35 and 27 MJ/kg DM on the feather and the fish meal diet, respectively. This degree of oversupply of ME implies that milk production would be relatively insensitive to an increase in the intake of

Table 3.12 Mammary enzyme activities, total DNA and udder volume in Experiment 2

	Period 1 (wk[4] 22)			Period 2 (wk 28)			Period 3 (wk 32)		
	C[1]	T[2]	SED[3]	C	T	SED	C	T	SED
Acetyl-CoA carboxylase, nmol/min per mg DNA	251	213	36.7	248	220	40.7	247	222	71.8
Fatty acid synthetase, μmol/min per mg DNA	0.88	0.84	0.145	0.72	0.53	0.089	0.85	0.70	0.126
Galactosyltransferase, μmol/min per mg DNA	0.62	0.48	0.096	0.50	0.40	0.037*	0.48	0.38	0.088
Total DNA, g	20.3	19.9	3.22	16.4	19.6	3.55	18.4	17.7	3.96
Udder volume, l	12.9	12.2	1.08	12.5	12.6	1.03	11.3	12.4	1.28

[1] Control group.
[2] Treatment group.
[3] SED, standard error of differences.
[4] Lactation week.
*P<0.05.

ME. This, in turn, would suggest that the large difference in milk production between the treatments would be very unlikely to be a result of the relatively modest difference in ME intake of 16 MJ/d. Moreover, this interpretation is supported by the results of previous experiments with the feather meal diet, in which intravenously infused amino acids induced large increases in milk production with little or no effect on ME intake (Choung and Chamberlain, 1995; Kim *et al.*, 1999; 2000; 2001a)

The difference in histidine provided by the UDP of fish meal and feather meal was around 11 g/d (Chamberlain *et al.*, 1992) which amounts to around 9 g/d of absorbable histidine (AFRC, 1992), which is roughly equivalent to the highest dose of histidine given intravenously by Kim *et al.* (2001a, b). When the cows returned to the fish meal diet, milk protein yield from the whole udder increased by 154 and 164 g/d in Experiment 1 and 2, respectively. Assuming that milk protein contains 26 g/kg of histidine (Kaufmann, 1980), these increases in protein yield correspond to efficiencies of transfer of histidine of 0.44 and 0.47 respectively, values that agree well with the estimate (0.41) obtained in short-term experiments in which histidine was given intravenously. Since the yield of milk protein declined progressively after changing to the amino acid-deficient diet, it is reasonable to infer that the cows mobilized reserves of amino acids to lessen the effect of the deficiency on milk production. This, in turn, means that when the animals were returned to the fish meal diet, their protein reserves were in a depleted state. It might be expected, therefore, that the extra amino acids provided by the fish meal would be partitioned between milk secretion and tissue synthesis (Whitelaw *et al.*, 1986) and that this might account for the apparently low transfer of histidine to milk.

Feather meal contains lower concentrations of His, Met, and Lys, and higher concentrations of branched-chain amino acids and Ser than fish meal (Chamberlain *et al.*, 1992). The profile of amino acids in blood plasma in both experiments reflected the

marked difference in the composition of amino acids between the two protein supplements. Although, in the present experiments, the significantly lower concentration of histidine in blood plasma with the feather meal diet in the second week of period 2 is consistent with a limitation of histidine with this diet, no difference was evident at the end of period 2. Changes in the plasma concentration of amino acids could arise from changes in the amounts of amino acids absorbed or from changes in the demands for these amino acids and hence are not completely reliable as an indicator of the limiting amino acid. The stage of lactation or the magnitude of milk yield could influence the demand for individual amino acids. Hence, in the present experiments, progressive decreases in the yields of milk protein with the feather meal diet might explain the lack of difference in the plasma concentrations of histidine between the two protein supplements at the end of period 2.

In relation to the increases in the concentration of milk fat with the feather meal diet, it has been suggested that an imbalance of amino acid supply, arising both from a surplus or a deficit of amino acid, might be responsible for the increases in milk fat secretion (Chamberlain *et al.*, 1992). Similarly, Cant *et al.* (2001) suggested that the protein: fat ratio of milk would be the most sensitive indicator of a circulating amino acid imbalance, being decreased with an imbalance of amino acid supply. Consistent with this, intravascular infusion of amino acid mixtures deficient in histidine markedly increased the yield and concentration of milk fat (Kim *et al.*, 1999; 2001a; Cant *et al.*, 2001) and reintroduction of histidine returned the fat concentration to the starting level. Interestingly, it has been suggested that an increase in mammary blood flow caused by a deficiency in histidine supply (Bequette *et al.*, 2000) might result in increases in delivery of milk fat precursors (Cant *et al.*, 1999). Whether the increased mammary blood flow is a response specifically to deficiency of histidine is not known but it is pertinent to note

that a similar tendency was observed during an experimentally imposed deficiency of leucine (Bequette *et al.*, 1996), raising the possibility that the effect might be more general. No measurements of mammary blood flow were made in the present experiments. However, despite marked decreases in the yields of protein and lactose in the feather meal diet in Experiment 1, the output of most of the fatty acids remained as high as that with the fish meal diet, suggesting a possible increase in mammary blood flow in the feather meal diet which might prevent a fall in delivery of milk fat precursors to the mammary gland.

With respect to the effects of amino acid supply on mammary function, the results show that indicators of mammary function were largely unaffected. In Experiment 1, significant differences in FAS and PCNA were seen only in period 1 when cows in both groups were given the control diet. This makes interpretation difficult. In Experiment 2, there was a significant difference in GT between the feather meal and the fish meal treatments at the end of period 2. However, the numerical difference of GT between two groups was already 23 % in period 1 and this difference seemed to change little throughout the experiment. Although positive relationships between udder volume and milk yield have been reported in goats (Linzell, 1966) and cows (Knight, 2000), the present results lend these no support. However, it should be noted that the measurement of udder volume might overestimate secretory tissue in the large, pendulous udders of older cows and underestimate it in heifers (Knight *et al.*, 1995). Little is known about the effects of nutrition on mammary function in the dairy cow. However, under similar dietary conditions, milk yield increases markedly, within 24 h, in response to intravenous infusions of the limiting amino acids (Kim *et al.*, 1999; 2000) and it would seem unlikely, in such a short time span, that changes in key enzyme activities and cell numbers in the mammary gland were responsible. Furthermore, despite its well known effects on

changing nutrient partitioning and milk production, bST does not affect the mRNA and protein abundance of acetyl-CoA carboxylase and fatty acid synthase in the mammary gland in dairy cows (Beswick and Kennelly, 1998). However, a recent study suggested that, under extreme dietary conditions, enzyme activities in the mammary gland can change in response to nutrient supply (Piperova *et al.*, 2000). In cows fed a diet containing a high ratio of concentrate to forage, a 45 % reduction in the yield and concentration of milk fat was seen, and FAS and ACC in the mammary gland were markedly decreased. Again, in cows fed two extreme levels of concentrate (25 vs 75 %) in the diet, the high concentrate diet increased PCNA in the mammary gland relative to the low concentrate diet (Sorensen *et al.*, 2000).

When examining the effect of a low-protein ration on milk yield, Oldham *et al.* (1979) reported that a low-protein diet decreased milk yield markedly, but they found a suggestion of over compensation in milk yield when changing back from low- to adequate-protein feeding. It was suggested that the over compensation might be due to increases in fibre digestion and energy intake on increasing the protein content in the diet. Although, in the present experiments, the fall of milk yield in the treatment group was completely recovered by a return to the control diet in period 3, no compensatory increases were observed.

CHAPTER FOUR

EFFECTS OF DIETARY AMINO ACID BALANCE ON THE RESPONSE TO AN INCREASE OF MILKING FREQUENCY FROM TWICE DAILY TO THRICE DAILY

4.1 INTRODUCTION

It is clear, from results of Chapter 3 that, in cows consuming a diet containing feather meal as the only protein supplement, milk production is limited by deficiencies in the supply of specific amino acids (His, Met and Lys). The supplies of total amino acids and metabolizable energy are both in substantial excess. So although, in theory, the cow is able to fuel a substantial increase of milk production by repartitioning energy towards the udder, it would seem that she cannot provide the extra limiting amino acids needed unless she mobilizes body protein. Since frequent milking is known to increase milk production, possibly via changing nutrient partitioning, it was thought to be a useful tool to test this hypothesis.

In the first experiment (Experiment 3), the responses of milk production to milking thrice daily were examined in cows in late lactation, consuming a diet containing feather meal as the only protein supplement. Since stage of lactation has been shown to affect the response to frequent milking (Blatchford and Peaker, 1982), the second experiment (Experiment 4) was performed in early lactation. Little response to frequent milking of the whole udder in the first experiment raised the possibility that compensatory changes occurred in the two udder halves. The use of half-udder milking might provide information on compensatory changes in milk yield between the two halves of the udder and hence this was adopted in the second experiment (Experiment 4).

Because of a lack of available cows at the time, it was difficult to include a positive dietary control in the first and the second experiments (Experiments 3 and 4). However, numerous studies show an increase of milk production in response to frequent milking (Poole, 1982; DePeters *et al.*, 1985; Henderson *et al.*, 1985; Wilde *et al.*, 1987a; Hillerton *et al.*, 1990; Dewhurst and Knight, 1994; Erdman and Varner, 1995; Sorensen and Knight, 1999) and thus it was assumed that positive responses to frequent milking in those studies could be a control for these two initial experiments. In the third experiment, fish meal, a positive control, was used against feather meal. Furthermore, since a deficiency in ME supply has been reported to inhibit an increase of milk production in response to frequent milking (Blatchford and Peaker, 1983), the response of frequent milking to an increase of ME supply was also investigated.

Because a metabolic index of body protein breakdown would considerably aid interpretation of the results of these experiments, a preliminary experiment was carried out to evaluate the use of spot-sampling of urine for estimation of excretion of 3-methylhistidine, a by-product of the degradation of actin and myosin.

4.2 EXPERIMENTAL

4.2.1 Animals and their management

A total of 12 Friesian cows in their second to fifth lactations were used in Experiments 3 and 4. They were 23 – 26 and 7 – 10 weeks into their lactation at the start of Experiments 3 and 4, respectively. In Experiment 5, six Friesian cows in their second lactation were used and the cows were 9 – 14 weeks into their lactation at the start of the experiment. Average body weight of the cows was approximately 576 kg (range 528 – 653 kg) in Experiment 3 and 568 kg (range 520 – 627 kg) in Experiment 4 and 534 kg (range 482 – 616 kg) in Experiment 5. The animals were housed in a metabolism cubicle

with water freely accessible. Silage was provided using an automatic feeder (RIC HF 2 PL, Insentec B. V., Marknesse, Netherlands) and concentrates were provided in two equal meals at milking times. The cows were milked twice daily at 07.00 and 15.00 h with an additional milking at 22.00 h for thrice-daily milking treatments.

In Experiments 3 and 4, all cows were milked twice daily and given *ad libitum* access to grass silage and 5 kg/d of feather meal (a pelleted mixture of 50 % rolled barley, 25 % feather meal and 25 % citrus pulp on a fresh weight basis) for 21 d before the start of the experiment. In experimental periods, the cows were given a basal diet consisting of a restricted amount of silage, which was 95 % of *ad libitum* intake in the introductory period, and 5 kg/d of feather meal. In Experiment 5, the basal diet consisted of a restricted amount of silage as described above and 3 kg/d of sugar beet pulp. For all experiments, the silage was made from perennial ryegrass (*Lolium perenne*) cut at an early stage of growth and ensiled with the addition of an inoculum of *Lactobacillus plantarum* (Ecosyl; ICL pcl, Billingham, UK) at 3 litres/tonne in a bunker silo of 70-tonne capacity. The chemical compositions of the silage and the supplements are shown in Table 4.1 and 4.2, respectively.

4.2.2 Experimental treatments and design

In a preliminary experiment, total urine from eight Friesian cows was collected via bladder catheter (silicone treated latex Foley catheter, 28Ch, 2-way male, 30 ml balloon, Folec, Malaysia) for 3 d to evaluate the use of spot-sampling of urine for estimation of 3-methylhistidine. During the total urine collection on the second and third day, spot urine was taken, using a syringe, from a silicone tube connected to the bladder catheter. Sampling times were 12.00, 15.00, 18.00 and 21.00 h. The ratio of 3-methylhistidine/creatinine was compared between spot-sampling times and the ratio

Table 4.1 The chemical composition (g/kg DM, unless stated otherwise) of the silages used in the experiments

	Exp. 3	Exp. 4	Exp. 5
DM (g/kg)	223	231	290
Organic matter	908	892	897
Total N	21.8	18.3	29.3
NPN (g/kg N)	751	672	752
Ammonia-N (g/kg N)	117	114	89
Water-soluble carbohydrate	24	7	38
pH	3.8	3.8	4.2
Lactic acid	132	98	93
DOMD[1]	647	611	687

[1] Digestible organic matter in the dry matter.

Table 4.2 The chemical composition (g/kg DM, unless stated otherwise) of the feather meal cube (FMC), fish meal cube (FC), and sugar beet pulp (SBP) used in the experiments

	Exp. 3	Exp. 4	Exp. 5		
	FMC	FMC	FMC	FC	SBP
DM (g/kg)	893	913	890	889	905
Organic matter	915	918	910	888	861
Total N	46.3	45.4	44.2	42.3	16.1
Starch	269	247	233	224	3
Sugars	64	78	95	44	205

from the spot urine was also compared with that in the total collection of urine.

4.2.2.1 Experiment 3

Experiment 3 consisted of three periods each of 21 d. In all periods cows were given the basal diet as described above. All cows were milked twice daily (2x) in period 1, were switched to thrice-daily milking (3x) in period 2 and were returned to twice-daily milking (2x) in period 3.

Food intake and milk yield were recorded daily. The composition of milk was determined on a representative, composite sample from the last four consecutive milkings in each experimental period. Samples of blood were taken from a tail vessel at 9:30 h on the last day of each period. Spot urine collections were made once on the last day of each period for determination of the ratio of 3-methylhistidine/creatinine.

4.2.2.2 Experiment 4

Experiment 4 consisted of three 14-d periods. In all periods cows were given the basal diet as described above. All quarters of the cows were milked twice daily in period 1. Then treatment glands (left hand side) were milked thrice daily in period 2 and returned to twice-daily milking in period 3. Control glands (right hand side) continued on twice-daily milking throughout the experiment.

Recording of food intake and milk yield were as described for Experiment 3. Samples of blood and urine were taken as in Experiment 3. Mammary blood flow (see Material and Methods, Chapter 2) was determined at 10.00 and 12.00 h on the last day of each period.

4.2.2.3 Experiment 5

Experiment 5 was designed as a 6 x 6 Williams Latin square with 14-d periods. The design is illustrated in Table 4.3. The dietary treatments were (1) the basal diet as described above plus 5 kg/d of feather meal (a pelleted mixture of 50 % rolled barley, 25 % feather meal and 25 % citrus pulp on a fresh weight basis) (FE); (2) the basal diet as described above plus 5 kg/d of fish meal (a pelleted mixture of 50 % rolled barley, 30 % fish meal and 20 % citrus pulp on a fresh weight basis) (FI); (3) FI plus 2 kg/d of sugar beet pulp (FI + ME). Under each dietary treatment, cows were milked twice (2x) or thrice daily (3x).

All animals were milked twice daily and given *ad libitum* access to grass silage and 5 kg/d of feather meal and 3 kg/d of sugar beet pulp for 14 d before the start of the experiment. Recording of food intake and milk yield were as described for Experiment 3. Samples of blood were taken as in Experiment 3. Mammary blood flow was determined at 10.00 h on the last day of each period.

4.2.3 Chemical analysis

Minced wet silage was analysed for DM by toluene distillation, total nitrogen, true protein, ammonia, lactic acid, water-soluble carbohydrate and pH. Dried samples of silage were analysed for ash and digestible organic matter in the dry matter (DOMD). Concentrate samples were analysed for DM, total nitrogen, ash, sugars and starch. Blood plasma samples were analysed for non-esterified fatty acids, glucose, and urea, and whole blood samples for total haemoglobin. Milk samples were analysed for fat, crude protein and lactose. Fatty acid composition of milk fat was analysed in Experiment 4. Urine samples were analysed for creatinine and 3-methylhistidine in Experiments 3 and 4.

4.2.4 Statistical analysis

Table 4.3 The experimental design of Experiment 5: a Williams Latin square

	Cows					
Period	**1**	**2**	**3**	**4**	**5**	**6**
I	A	B	C	D	E	F
II	F	A	B	C	D	E
III	B	C	D	E	F	A
IV	E	F	A	B	C	D
V	C	D	E	F	A	B
VI	D	E	F	A	B	C

Treatments: A, FI 2x; B, FI + ME 3x; C, FI 3x; D, FE 2x; E, FE 3x; F, FI + ME 2x.

For statistical analysis, mean values for feed intake and milk yield were taken for the last 7 d of each experimental period. In Experiment 4, in period 1, one of the cows had a high milk somatic cell count and did not recover until the end of experiment. Consequently, all the data for this animal were excluded from the statistical analysis. In Experiment 5, one of the cows became lame during the fourth period, and her silage intake and milk production were depressed. Although she recovered well during the rest of the experiment, the data for this cow for that period were excluded from the statistical analysis. In Experiment 3, mean values of variables in periods 1 and 3 were compared with those in period 2 by paired *t*-test. In Experiment 4, treatment differences in milk production and mammary blood flow between two half-udders were compared for period 2 and 3 separately by analysis of covariance with the period 1 response as a covariate, using the directives of Genstat 5 (Lawes Agricultural Trust, 1990). For feed intake, body weight, metabolites in blood plasma and in urine, treatment differences were compared as described for Experiment 3 and this statistical method was also used to examine compensatory responses in milk production. Results of Experiment 5 were analysed by ANOVA using the directives of Genstat 5. All differences between treatment means were inspected by the least significant difference (LSD) method at the 5 % level. The statistical models used were as follows:

Y = Overall mean + Cow + Treatment + Error (Experiment 3)

Y = Overall mean + Cow + Treatment + b(covariate – covariate mean) + Error (Experiment 4) where values for both halves were available; otherwise, the model was as for Experiment 3.

Y = Overall mean + Cow + Period + Treatment + Error (Experiment 5)

4.3 RESULTS

4.3.1 Preliminary experiment

There were no significant differences in the ratio of 3-methylhistidine/creatinine in spot urine samples between times during the day (Table 4.4). A significant relationship between the concentration of 3-methylhistidine (μmol/l) and that of creatinine (mmol/l) ($r^2 = 0.82$; $n = 8$; $P<0.01$) in the spot urine was found. To illustrate the effect of time, each measurement of the ratio of 3-methylhistidine/creatinine from the spot urine was expressed as a proportion of the daily mean of that from the corresponding animal and the result is shown in Figure 4.1. There were no significant differences in the ratio of 3-methylhistidine/creatinine between the spot samples and the total urine.

4.3.2 Experiment 3

Results for feed intake and milk production in Experiment 3 are shown in Table 4.5. There was a very small, but significant difference in silage intake between the treatments. The 3x milking did not affect milk yield. Neither the concentration nor the yield of milk protein and lactose was affected by the 3x milking. However, the 3x milking increased the fat concentration ($P<0.05$) and tended to increase fat yield ($P<0.10$).

The concentration of haemoglobin in blood was significantly higher for the 3x milking than for the 2x milking (Table 4.6). There were no significant differences between the treatments in the ratio of 3-methylhistidine to creatinine in urine.

4.3.3 Experiment 4

Results for feed intake and body weight in Experiment 4 are shown in Table 4.7. Silage intake was not affected by treatment. Body weight tended to be lower for the treatment than for the control.

Milk production and mammary blood flow are shown in Table 4.8. There were no

Table 4.4 Effect of time of spot sampling on urinary 3-methylhistidine/creatinine ratio (μmol/mmol). (The ratio in total urine collection was 14.8 ± 0.57)

	Time (h)					
	12.00	15.00	18.00	21.00	SED[1]	P value
3-methylhistidine/ creatinine ratio	15.9	14.7	15.4	14.0	1.08	0.311

[1] SED, standard error of differences.

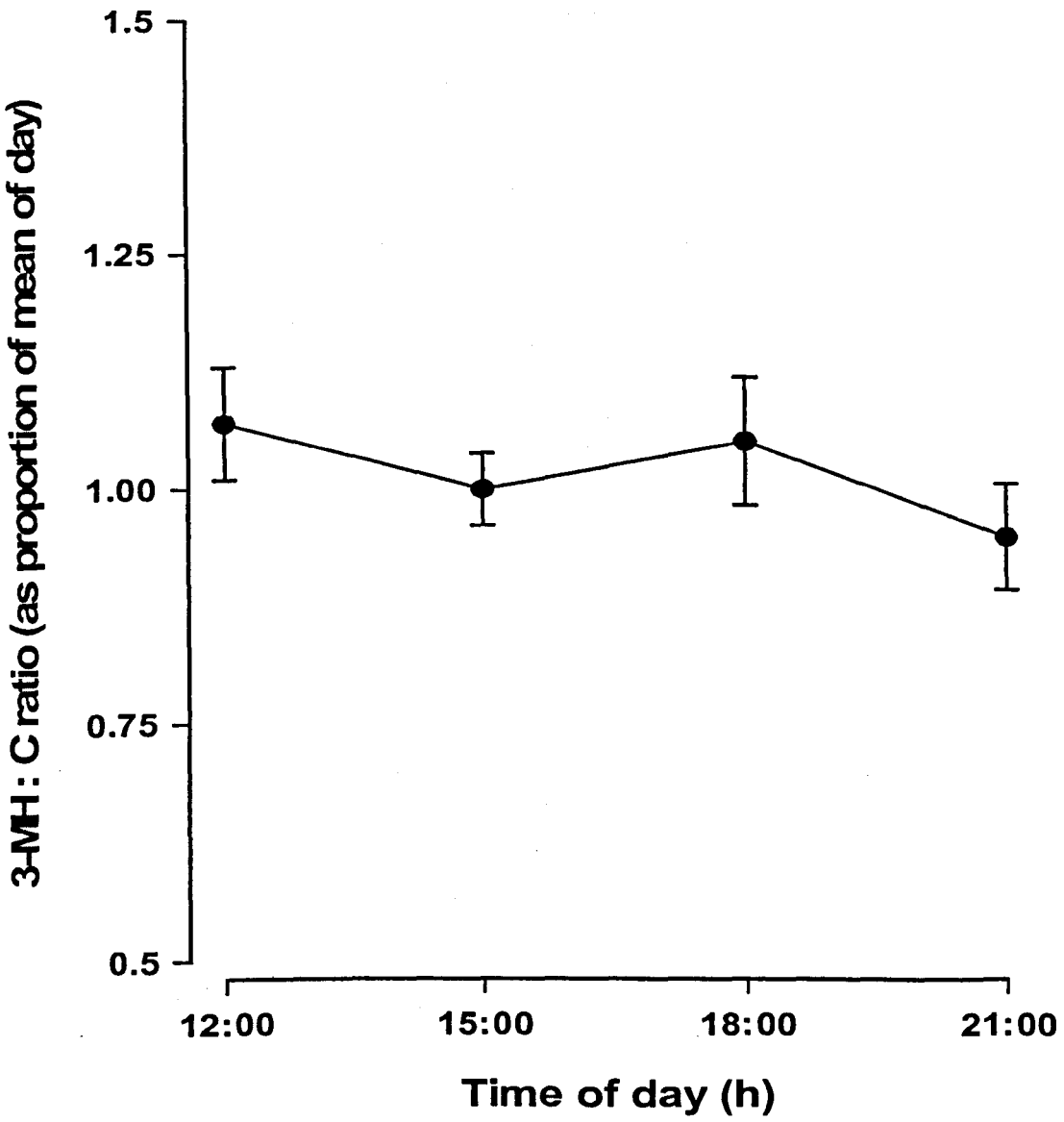


Figure 4.1 Diurnal changes in the ratio (μmol/mmol) of 3-methylhistidine/creatinine (3-MH : C) in spot urine. The value of each time is expressed as a proportion of the mean of the day determined from analysis of total collection of urine (Mean ± s.e.m. of 8 cows).

Table 4.5 Feed intake and milk production in Experiment 3

	2x[2]	3x	SED[1]	P value
DM intake, kg/d				
Silage	10.2	10.3	0.04	0.007
Total	14.6	14.8	0.04	0.006
Milk yield, kg/d	16.5	16.8	0.34	0.450
Milk protein, g/kg	33.0	32.7	0.39	0.523
g/d	539	541	8.7	0.873
Milk fat, g/kg	48.4	51.2	1.07	0.047
g/d	780	839	25.6	0.068
Milk lactose, g/kg	45.0	45.5	0.51	0.451
g/d	743	765	23.5	0.395

[1] SED, standard error of differences.
[2] 2x is the mean of Periods 1 and 3.

Table 4.6 The concentration (g/l) of haemoglobin in blood and the ratio (μmol/mmol) of 3-methylhistidine/creatinine in urine in Experiment 3

	2x[2]	3x	SED[1]	P value
Haemoglobin	90	98	1.7	0.005
3-methylhistidine/ creatinine ratio	12.8	13.7	1.22	0.519

[1] SED, standard error of differences.
[2] 2x is the mean of Periods 1 and 3.

Table 4.7 Feed intake and body weight in Experiment 4

	Control[2]	Treatment	SED[1]	P value
DM intake, kg/d				
Silage	9.4	9.0	0.22	0.137
Total	14.0	13.6	0.22	0.137
Body weight, kg	550	536	5.8	0.071

[1] SED, standard error of differences.
[2] Control is the mean of Periods 1 and 3.

Table 4.8 Milk production and mammary blood flow in Experiment 4

	Period 1				Period 2				Period 3			
	C[1]	T[2]	SED[3]	P value	C	T	SED	P value	C	T	SED	P value
Milk yield, kg/d	9.8	9.9	0.41	0.866	8.6	9.1	0.16	0.059	8.4	8.4	0.32	0.964
Milk protein, g/kg	27.4	27.3	0.13	0.598	27.7	27.6	0.27	0.884	30.3	30.1	0.42	0.687
g/d	265	264	11.3	0.926	234	247	5.68	0.111	247	249	11.1	0.909
Milk fat, g/kg	37.0	38.8	1.40	0.303	36.6	44.3	1.96	0.029	37.5	38.9	2.00	0.553
g/d	360	367	26.5	0.794	310	385	15.4	0.017	308	315	17.5	0.713
Milk lactose, g/kg	46.0	46.1	0.11	0.230	45.6	46.5	0.28	0.056	46.5	46.6	0.18	0.756
g/d	447	455	17.0	0.668	393	424	5.2	0.009	389	393	12.0	0.782
Mammary blood flow, l/min	7.9	6.6	0.34	0.019	8.1	7.1	1.01	0.389	9.7	6.8	1.91	0.221

[1] Control half-udder.
[2] Treatment half-udder
[3] SED, standard error of differences.

no significant differences in milk production between the two half-udders in periods 1 and 3 when both half-udders were milked twice daily. Although milk yield tended to be slightly higher for the treatment glands than for the control glands in period 2, this seemed to be caused by a fall in milk yield in the control glands. The concentration and yield of milk protein were not affected by thrice-daily milking in period 2. Yields of milk fat and lactose were significantly higher for the treatment glands than for the control glands in period 2. The concentrations of milk fat ($P<0.05$) and lactose ($P<0.10$) of the glands milked thrice daily increased compared with those milked twice daily. There was a significant difference in mammary blood flow between the two half-udders in period 1.

To identify the cause of the difference in milk yield between the two half-udders, mean values for milk production in periods 1 and 3 were compared with those in period 2 in each half-udder (Table 4.8.a, b). Compared with the mean of periods 1 and 3, little change in milk yield was seen in the glands milked thrice daily in period 2. However, milk yield of the control glands in period 2 was significantly decreased compared with the mean of periods 1 and 3, indicating that the higher milk yield from the glands milked thrice daily was caused by a decrease in milk yield from the glands milked twice daily (Table 4.8). This is true also for the yields of milk protein and lactose. However, the yields of milk fat showed a different response. In the control glands, there was no significant difference in the yield of milk fat between the periods whereas fat yield in the treatment glands in period 2 was significantly increased relative to the mean of periods 1 and 3.

The concentrations and yields of milk fatty acids are shown in Table 4.9 and 4.10. In period 2, there was a small, but significant decrease in the concentration of $C_{14:0}$ in the treatment glands compared with that in the control glands. With the exception of $C_{6:0}$ and

Table 4.8. Comparison of the mean of periods 1 and 3 with the value of period 2 in the control glands (a) and the treatment glands (b)

(a) Control glands

	Mean of periods 1 and 3	Period 2	SED[1]	P value
Milk yield, kg/d	9.2	8.7	0.16	0.041
Milk protein, g/d	259	237	6.3	0.023
Milk fat, g/d	333	305	22.0	0.269
Milk lactose, g/d	426	399	9.3	0.043

[1]SED, standard error of differences.

(b) Treatment glands

	Mean of periods 1 and 3	Period 2	SED[1]	P value
Milk yield, kg/d	9.0	9.0	0.13	0.873
Milk protein, g/d	254	245	3.9	0.090
Milk fat, g/d	342	390	15.5	0.036
Milk lactose, g/d	416	418	6.2	0.774

[1]SED, standard error of differences.

Table 4.9 The fatty acid composition (g/kg) of milk fat in Experiment 4

	Period 1				Period 2				Period 3			
	C[1]	T[2]	SED[3]	P value	C	T	SED	P value	C	T	SED	P value
$C_{4:0}$	91	103	7.7	0.222	60	73	5.2	0.093	69	74	3.1	0.231
$C_{6:0}$	30	32	1.3	0.365	26	26	0.9	0.473	29	30	1.3	0.515
$C_{8:0}$	13	12	0.3	0.742	12	12	0.3	0.060	14	14	0.5	0.975
$C_{10:0}$	19	23	6.1	0.576	22	23	1.1	0.491	30	30	0.8	0.409
$C_{12:0}$	24	26	1.7	0.243	26	25	0.6	0.365	33	35	0.5	0.080
$C_{14:0}$	92	97	5.2	0.380	98	95	0.9	0.028	118	121	4.0	0.513
$C_{4:0}$ - $C_{14:0}$	271	292	20.3	0.390	245	255	9.2	0.376	289	308	14.5	0.293
$C_{16:0}$	332	345	13.9	0.427	357	346	4.5	0.083	397	430	9.8	0.043
$C_{16:1}$	28	29	4.0	0.861	26	24	2.0	0.488	24	22	2.7	0.572
$C_{16:0}$ - $C_{16:1}$	358	376	18.4	0.407	383	370	5.5	0.110	419	454	14.0	0.093
$C_{18:0}$	106	101	4.1	0.280	108	110	5.3	0.659	93	85	1.0	0.003
$C_{18:1}$	258	230	20.8	0.268	264	256	3.8	0.113	180	165	20.8	0.512
$C_{18:0}$ - $C_{18:1}$	365	331	11.2	0.056	371	368	5.0	0.649	268	254	19.7	0.536

[1] Control half-udder.
[2] Treatment half-udder.
[3] SED, standard error of differences.

Table 4.10 The yield of fatty acids (g/d) in milk fat in Experiment 4

	Period 1				Period 2				Period 3			
	C[1]	T[2]	SED[3]	P value	C	T	SED	P value	C	T	SED	P value
$C_{4:0}$	35	37	7.9	0.848	19	28	2.1	0.024	21	23	1.8	0.374
$C_{6:0}$	11	12	1.3	0.676	8	10	0.6	0.070	9	10	0.7	0.537
$C_{8:0}$	5	5	0.3	0.883	4	4	0.2	0.140	4	5	0.3	0.549
$C_{10:0}$	7	9	1.8	0.491	7	9	0.4	0.022	9	9	0.6	0.776
$C_{12:0}$	9	10	0.6	0.276	8	10	0.4	0.037	10	11	0.7	0.605
$C_{14:0}$	33	36	1.9	0.225	30	37	2.0	0.033	37	38	1.8	0.560
$C_{4:0}$ - $C_{14:0}$	109	116	4.6	0.214	76	98	4.9	0.022	91	95	5.9	0.488
$C_{16:0}$	118	127	4.3	0.135	111	134	4.0	0.010	125	134	10.1	0.464
$C_{16:1}$	10	11	0.7	0.750	8	10	0.4	0.023	7	7	0.4	0.184
$C_{16:0}$ - $C_{16:1}$	128	139	4.9	0.111	118	145	5.2	0.013	132	141	10.0	0.458
$C_{18:0}$	38	37	2.4	0.696	33	42	2.4	0.033	29	27	1.6	0.320
$C_{18:1}$	91	86	7.4	0.619	82	98	2.9	0.011	53	53	2.2	0.955
$C_{18:0}$ - $C_{18:1}$	129	123	8.6	0.522	115	141	4.6	0.012	82	80	3.4	0.601

[1] Control half-udder.
[2] Treatment half-udder.
[3] SED, standard error of differences.

$C_{8:0}$, the yields of all fatty acids were significantly increased by thrice-daily milking in period 2.

There were no significant differences between the treatments in the composition of blood plasma, or in the ratio of 3-methylhistidine/creatinine in urine (Table 4.11).

4.3.4 Experiment 5

Results for feed intake, milk production and mammary blood flow are given in Table 4.12. As silage intake in the FI and the FI + ME treatments was restricted to a level of that in the FE treatment, silage intake between the treatments remained similar. However, as a result of an additional supply of 2 kg/d of sugar beet pulp, the FI + ME treatment significantly increased total intake relative to the other treatments. Compared with the 2x milking, the 3x milking significantly increased milk yield in the FI and the FI + ME treatments but did not affect it on the FE treatment. Regardless of milking frequencies, milk yields were significantly higher for the FI and the FI + ME treatments than for the FE treatment. Within a milking frequency, there were no significant differences in milk yields between the FI and the FI + ME treatments. In a comparison within each dietary treatment, the concentrations of milk protein, fat and lactose were not affected by the 3x milking with the exception that there was a significant difference in the concentration of milk lactose between the 2x and the 3x milkings in cows fed the feather meal diet. In both twice and thrice-daily milking, the FI and the FI + ME treatments significantly increased the concentration of milk protein but decreased that of milk fat relative to the FE treatment. The 3x milking did not affect the yields of milk protein, fat and lactose in cows fed the feather meal diet compared with the 2x milking. However, in cows fed the fish meal diets, the yields of milk fat and lactose were significantly higher

Table 4.11 The composition of plasma and blood, and the ratio (μmol/mmol) of 3-methylhistidine/creatinine in urine in Experiment 4

	Control[2]	Treatment	SED[1]	P value
Glucose, mmol/l	3.64	3.43	0.196	0.344
Urea – N, mmol/l	4.93	4.86	0.082	0.384
Haemoglobin, g/l	87	88	0.8	0.457
NEFA[3], mmol/l	0.03	0.03	0.009	0.898
3-methylhistidine/ creatinine ratio	10.8	12.3	1.08	0.232

[1] SED, standard error of differences.
[2] Control is the mean of Periods 1 and 3.
[3] Non-esterified fatty acids.

Table 4.12 Feed intake, milk production and mammary blood flow in Experiment 5

	FE		FI		FI + ME			
	2x	3x	2x	3x	2x	3x	SED[1]	P value[2]
DM intake, kg/d								
Silage	12.0	11.9	12.2	12.2	12.0	12.0	0.17	0.439
Total	19.2^{a}	19.0^{a}	19.3^{a}	19.4^{a}	21.0^{b}	20.9^{b}	0.17	<0.001
Milk yield, kg/d	21.7^{a}	22.3^{a}	25.0^{bc}	26.6^{d}	24.3^{b}	26.0^{cd}	0.76	<0.001
Milk protein, g/kg	33.3^{a}	34.3^{a}	35.9^{b}	35.6^{b}	36.3^{b}	36.2^{b}	0.51	<0.001
yield, g/d	722^{a}	764^{a}	896^{bc}	944^{c}	881^{b}	938^{bc}	29.4	<0.001
Milk fat, g/kg	44.2^{b}	42.5^{b}	35.8^{a}	38.3^{a}	37.8^{a}	38.2^{a}	1.56	<0.001
yield, g/d	954^{abc}	935^{ab}	891^{a}	1014^{c}	908^{a}	990^{bc}	33.0	0.010
Milk lactose, g/kg	48.2^{b}	47.3^{a}	46.6^{a}	47.0^{a}	47.0^{a}	47.0^{a}	0.35	0.007
yield, g/d	1046^{a}	1053^{a}	1166^{bc}	1250^{d}	1142^{b}	1221^{cd}	31.8	<0.001
Mammary blood flow, l/min	14.8	14.8	13.7	14.3	13.2	14.0	0.98	0.562

[1] SED, standard error of differences.
[2] Statistical significance of treatment effects by *F*-test.
[a, b, c, d] Means in the same row with unlike superscripts differ significantly (P<0.05) by *t*-test.

for the 3x milking than for the 2x milking. There were no significant differences in mammary blood flow between the treatments.

The composition of plasma and blood are shown in Table 4.13. In cows fed the feather meal diet, the concentration of haemoglobin in blood was lower for the 3x milking than for the 2x milking.

4.4 Discussion

In the present experiments, silage intake was restricted to 95 % of *ad libitum* intake in the introductory period, to avoid differences in ME supply between the FE and the FI treatments. The results clearly showed that, in cows fed a diet containing feather meal as the only protein supplement, frequent milking had little or no effect on milk secretion in the two stages of lactation. Although no positive dietary control was included in Experiments 3 and 4 owing to the lack of available cows at the time, the results of Experiment 5 confirmed that, with the feather meal diet, milk yield was not affected by thrice-daily milking. However, with the fish meal diet, a positive control, milk yield increased in response to thrice-daily milking. Moreover, it would seem unlikely that increased milk yield in response to frequent milking in the present experiment was due to energy supply. In Experiment 5, silage intake between the FE and the FI treatments remained similar and, with additional ME supply in the FI + ME treatment, no increase in milk yield was seen for either twice or thrice-daily milking. Little is known about the effects of nutrient supply, in particular protein and amino acids, on responses to frequent milking. Although considerable variation in the response of milk secretion to frequent milking was seen in studies (Linzell, 1967; Linzell and Peaker, 1971) where dietary input was not standardized, the review of Erdman and Varner (1995) reported that increases in milk yield, in response to increasing milking frequency from two to three times daily, were relatively fixed (around 3.5 kg/d). However, where energy supply was limited by

4.13 The composition of plasma and blood in Experiment 5

	FE		FI		FI + ME			
	2x	3x	2x	3x	2x	3x	SED[1]	P value[2]
Haemoglobin, g/l	93[b]	86[a]	97[b]	95[b]	92[ab]	95[b]	3.0	0.032
Glucose, mmol/l	3.81	3.58	3.94	3.68	3.92	3.73	0.177	0.325
Urea-N, mmol/l	5.43	5.07	6.00	5.89	5.07	5.29	0.439	0.201

[1] SED, standard error of differences.
[2] Statistical significance of treatment effects by *F*-test.
[a, b, c, d] Means in the same row with unlike superscripts differ significantly (P<0.05) by *t*-test.

fasting (Linzell, 1967) or restricting feed intake (Blatchford and Peaker, 1983), milk secretion fell markedly and no response to hourly milking was seen.

As regards effects of stage of lactation on the response to frequent milking, it has been shown that, in goats, hourly milking did not affect milk secretion at peak or in late lactation (Blatchford and Peaker, 1982). However, in a later experiment, an increase of milk yield was observed in goats milked thrice daily in late lactation as well as in early lactation (Henderson *et al.*, 1983). In long-term experiments, in cows, the response to thrice-daily milking tended to be lower in early lactation than that in mid- to -late lactation (Pelissier *et al.*, 1978; Pearson *et al.*, 1979; Amos *et al.*, 1985; DePeters *et al.*, 1985). This might be partly explained by the fact that cows were in negative energy balance in early lactation (Pearson *et al.*, 1979).

Since little response to frequent milking of the whole udder was seen in Experiment 3, the possibility was considered that whole udder milking might mask compensatory changes in milk yield between udder halves. In Experiment 4, compensatory responses were examined (Table 4.8a, b) and it would seem that a small, but significant decrease in milk yield from the glands milked twice daily contributed to the apparent higher milk yield in the glands milked thrice daily (Table 4.8), suggesting nutrient repartitioning between the two half-udders. This agrees with a previous study where the increase of milk yield in one half-udder in response to thrice-daily milking was accompanied by a small, but significant decrease of milk yield in the other half-udder, which was milked twice daily (Dewhurst and Knight, 1994). It was suggested that the increased nutrient demand from the glands milked thrice daily might result in a reduction in nutrient supply to the glands milked twice daily (Dewhurst and Knight, 1994).

Whether increases in nutrient supply to the mammary gland arose from a change in mammary blood flow cannot be answered by the results. However, the response of milk

yield to frequent milking with the feather meal diet was very small and, even with the fish meal diets, the mean increase of milk yield was only 7 %. Proportionate increases in mammary blood flow would be difficult to detect, given the sensitivity of the technique.

To examine the sensitivity of the measurement of mammary blood flow, mammary blood flow from both sides of the udder in a lactating cow was determined hourly for six hours during the day. Mean coefficients of variation from both sides of the udder was 12 %, which is less than that from Christensen *et al.* (1989), in which the corresponding value was 17 % in a lactating goat. Furthermore, in the present study, the increase of mammary blood flow during milking was determined and was 27 %, agreeing well with the report of Metcalf *et al.* (1992), in which the corresponding figure was 25 %. Although the sensitivity of the method used in the present studies is similar to that reported in other studies, it is unlikely that the current method would be sensitive enough to detect such a small increase in milk yield. The least significant difference (LSD), expressed as the proportion of the mean of the two treatments, was calculated based on an equation reported by Chen *et al.* (1992) and was 0.15 (n = 6).

It has been suggested that urinary excretion of 3-methylhistidine could be used as a measure of muscle protein breakdown in cattle (Harris and Milne, 1981; McCarthy *et al.*, 1983; Gopinath and Kitts, 1984) and lactating cows (Harris *et al.*, 1980). During the catabolism of myofibrillar proteins, 3-methylhistidine is released and excreted in urine and is not reutilized for protein synthesis (Young *et al.*, 1972) or metabolized oxidatively (Long *et al.*, 1975). In the present studies, the ratio of 3-methylhistidine/creatinine was used to indicate the rate of degradation of muscle protein relative to the muscle mass of the animal (McCarthy *et al.*, 1983; Simmons *et al.*, 1994). The concentration of 3-methylhistidine showed similar changes with time to those seen for creatinine and no significant differences in the ratio of 3-methylhistidine/creatinine between the spot-

sampling times were observed. The coefficient of variation in the ratio of 3-methylhistidine/creatinine between sampling times was .16 %. This indicates that the LSD, calculated as described above, is relatively high (LSD = 0.21; n = 6). The magnitude of LSD indicates that the use of 3-methylhistidine/creatinine in spot samples would be suitable for detecting only relatively large differences between treatments.

Although body weight changes can only be a rough guide to changes of nutrient partitioning, cows milked thrice daily tend to gain less body weight than those milked twice daily (Pearson *et al.*, 1979; Amos *et al.*, 1985; DePeters *et al.*, 1985), indicating a possible role of frequent milking in changing nutrient partitioning. Therefore, it was assumed that, when the supply of specific amino acids was deficient, increase of milk yield in response to frequent milking would be accompanied by repartitioning of amino acid use between udder and body or by body protein mobilization. In cows fed the feather meal diet, the effect of frequent milking on nutrient partitioning between the body and the mammary glands would appear to be slight. However, nutrient repartitioning between the two half-udders was observed. Little increase in milk yield in response to frequent milking with the feather meal diet raises the question whether a stronger stimulus to nutrient partitioning, such as that provided by growth hormone, might provide extra amino acids for milk production so allowing an increase of milk yield in response to frequent milking.

CHAPTER FIVE

EFFECTS OF INJECTION OF GROWTH HORMONE ON THE RESPONSE TO AN INCREASED MILKING FREQUENCY IN COWS GIVEN DIETS OF DIFFERENT AMINO ACID COMPOSITIONS

5.1 INTRODUCTION

Results of experiments in Chapter 4 show that, in cows given a diet containing feather meal as the only protein supplement, more frequent milking had little or no effect on milk secretion. However, when cows were given fish meal, milking more frequently clearly increased milk yield, suggesting that the response to an increase of milking frequency can be altered by nutrient supply, particularly amino acids. Although, in cows fed the feather meal diet, nutrient repartitioning between the two half-udders occurred in response to thrice-daily milking, the effect of frequent milking on nutrient partitioning between the body and the mammary glands would appear to be slight. In the present study, it was decided to investigate whether growth hormone, a potent re-partitioning agent, might provide extra amino acids for milk production so allowing an increase of milk yield in response to frequent milking.

5.2 EXPERIMENTAL

5.2.1 Animals and their management

Eight Friesian cows in their second lactation were used in Experiment 6. They were 9 – 10 weeks into their lactation at the start of the experiment and weighed, on average, 568 kg (range 513 – 618 kg). The animals were housed in a metabolism cubicle with water freely accessible. Silage was provided using an automatic feeder (RIC HF 2 PL, Insentec B. V., Marknesse, Netherlands) and concentrates were provided in two equal

meals at milking times. The cows were milked twice daily at 07.00 and 15.00 h with an additional milking at 22.00 h for thrice-daily milking treatments.

Cows were given a basal diet consisting of *ad libitum* access to grass silage and 4 kg/d of sugar beet pulp. Silage was made from perennial ryegrass (*Lolium perenne*) cut at an early stage of growth and ensiled with the addition of an inoculum of *Lactobacillus plantarum* (Ecosyl; ICL pcl, Billingham, UK) at 3 litres/tonne in a bunker silo of 70-tonne capacity. The chemical compositions of the silage and the supplements are shown in Table 5.1 and 5.2, respectively.

5.2.2 Experimental treatments and design

The experiment was designed as a duplicated 4 x 4 Williams Latin square with two blocks of animals. Cows were blocked, on the basis of milk yield, into two groups at the start of the experiment. Each block contained 4 animals, 4 treatments and four 28-d periods. The design is illustrated in Table 5.3. The experimental treatments were (1) the basal diet as described above plus 5 kg/d of feather meal (a pelleted mixture of 50 % rolled barley, 25 % feather meal and 25 % citrus pulp on a fresh weight basis) (FE); (2) FE with administration of recombinant bovine growth hormone (bST, 30 mg/d, a daily intramuscular injection, Monsanto, St. Louis, USA) (FE + bST); (3) the basal diet as described above plus 5 kg/d of fish meal (a pelleted mixture of 50 % rolled barley, 30 % fish meal and 20 % citrus pulp on a fresh weight basis) (FI); (4) FI with administration of bST as described above (FI + bST). For the first 14 d of each period, all quarters of the cows were milked twice daily. For the rest of the period, one half-udder (left hand side) was milked thrice daily (3x) and the other half (right hand side) remained on twice-daily milking (2x).

Table 5.1 The chemical composition (g/kg DM, unless stated otherwise) of the silage used in Experiment 6

DM (g/kg)	281
Organic matter	909
Total N	21.8
NPN (g/kg N)	703
Ammonia-N (g/kg N)	97
Water-soluble carbohydrate	32
pH	3.9
Lactic acid	83
DOMD[1]	648

[1] Digestible organic matter in the dry matter.

Table 5.2 The chemical composition (g/kg DM, unless stated otherwise) of the feather meal cube (FMC), fish meal cube (FC) and sugar beet pulp (SBP) used in Experiment 6

	FMC	FC	SBP
DM (g/kg)	881	877	907
Organic matter	910	888	881
Total N	45.1	47.5	17.0
Starch	266	264	1
Sugars	108	43	264

Table 5.3 The experimental design of Experiment 6: a duplicated Williams Latin square

	Cows – group 1				Cows – group 2			
Period	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
I	A	B	D	C	A	B	D	C
II	B	D	C	A	D	C	B	A
III	C	A	B	D	C	D	A	B
IV	D	C	A	B	B	A	C	D

Treatments: A, FI + bST; B, FE; C, FE + bST; D, FI

Food intake and milk yield were recorded daily. The composition of milk was determined on a representative, composite sample from the last four consecutive milkings in the second week and in the last week of each experimental period. Samples of blood were taken from a tail vessel at 11:00 h on the last day of each period. Spot urine collections were made once on the last day of each period for determination of the ratio of 3-methylhistidine/creatinine. Mammary blood flow was determined at 10.00 h on the penultimate day and on the last day of each period, for cows in group 1 and 2 respectively.

5.2.3 Chemical analysis

Minced wet silage was analysed for DM by toluene distillation, total nitrogen, true protein, ammonia, lactic acid, water-soluble carbohydrate and pH. Dried samples of silage were analysed for ash and digestible organic matter in the dry matter (DOMD). Concentrate samples were analysed for DM, total nitrogen, ash, sugars and starch. Blood plasma samples were analysed for non-esterified fatty acids, glucose, urea, free AAs, growth hormone, IGF-I and insulin and whole blood samples for total haemoglobin. Milk samples were analysed for fat, crude protein and lactose. Urine samples were analysed for creatinine and 3-methylhistidine.

5.2.4 Statistical analysis

For statistical analysis, mean values for feed intake and milk yield from each half-udder were taken for the last 7 d of each experimental period. An additional mean value for milk yield from each half-udder was taken for the last 7 d of the second week in each period. One of the cows in group 2 had a high body temperature during the third period, and her silage intake and milk production were depressed. She recovered well during the

rest of the experiment, but the data for this cow for period 3 were excluded from the statistical analysis. Results from the last week of each experimental period were analysed by ANOVA using model a. This model was used to test for difference between FI and FE, presence and absence of bST, and any possible interaction between them. Differences due to milking frequency and its interaction with other factors was investigated by ANCOVA (see model b) where the response for the second week in each period was used as the covariate. Where variance ratio tests were statistically significant at the 5 % level, further investigation of treatment mean differences was undertaken using Fisher's least significant difference method (LSD) at the 5 % level.

(a) $Y = \mu + b_i + a_{i(j)} + bc_{ik} + d_l + h_m + dh_{lm} + ac_{i(jk)} + m_n + dm_{ln} + hm_{mn} + dhm_{lmn} + e_{ijklmn}$

(b) $Y = \mu + b_i + a_{i(j)} + bc_{ik} + d_l + h_m + dh_{lm} + ac_{i(jk)} + m_n + dm_{ln} + hm_{mn} + dhm_{lmn} + e_{ijklmn} + f(\text{covariate} - \text{covariate mean})$

where μ = overall mean

b_i = block

$a_{i(j)}$ = cow in block

bc_{ik} = block x period interaction

d_l = diet level

h_m = bST level

dh_{lm} = diet level x bST level interaction

$ac_{i(jk)}$ = residual error term for diet, bST and diet x bST interaction

m_n = milking frequency level

dm_{ln} = diet level x milking frequency level interaction

hm_{mn} = bST level x milking frequency level interaction

dhm_{lmn} = diet level x bST level x milking frequency level interaction

e_{ijklmn} = residual error

f = covariate coefficient

5.3 RESULTS

Milk yields from the whole udder in Experiment 6 are shown in Figure 5.1. There were significant differences in milk yield between the treatments. Regardless of the bST treatments, milk yield was significantly higher for the fish meal diet than for the feather meal diet. For both diets, bST significantly increased milk yield. Therefore, the highest milk yield was seen in the FI + bST treatment and the lowest was seen in the FE treatment.

Results for feed intake, body weight, milk production and mammary blood flow from the whole udder are given in Table 5.4. DM intake was not affected by the bST treatment. However, silage and total intakes were reduced by the feather meal diet. As regards the main effects of diet and bST on milk composition, bST significantly increased the concentrations of milk fat and lactose but decreased that of milk protein. Yields of milk protein, fat and lactose were significantly higher for the bST treatment than for the control. The fish meal diet significantly increased the concentration of milk protein but decreased that of milk fat compared with the feather meal diet. Yields of milk protein, fat and lactose were significantly higher for the fish meal diet than for the feather meal diet.

Within each dietary treatment, bST decreased the concentrations of milk protein but increased that of milk lactose for both diets. However, bST increased the concentration of milk fat only for the FE treatment. In both diets, bST increased yields of milk protein, fat and lactose. Within the controls, although there were no significant differences in the concentrations of milk protein, fat and lactose between the two diets, yields of milk protein, fat and lactose were significantly higher for the fish meal diet.

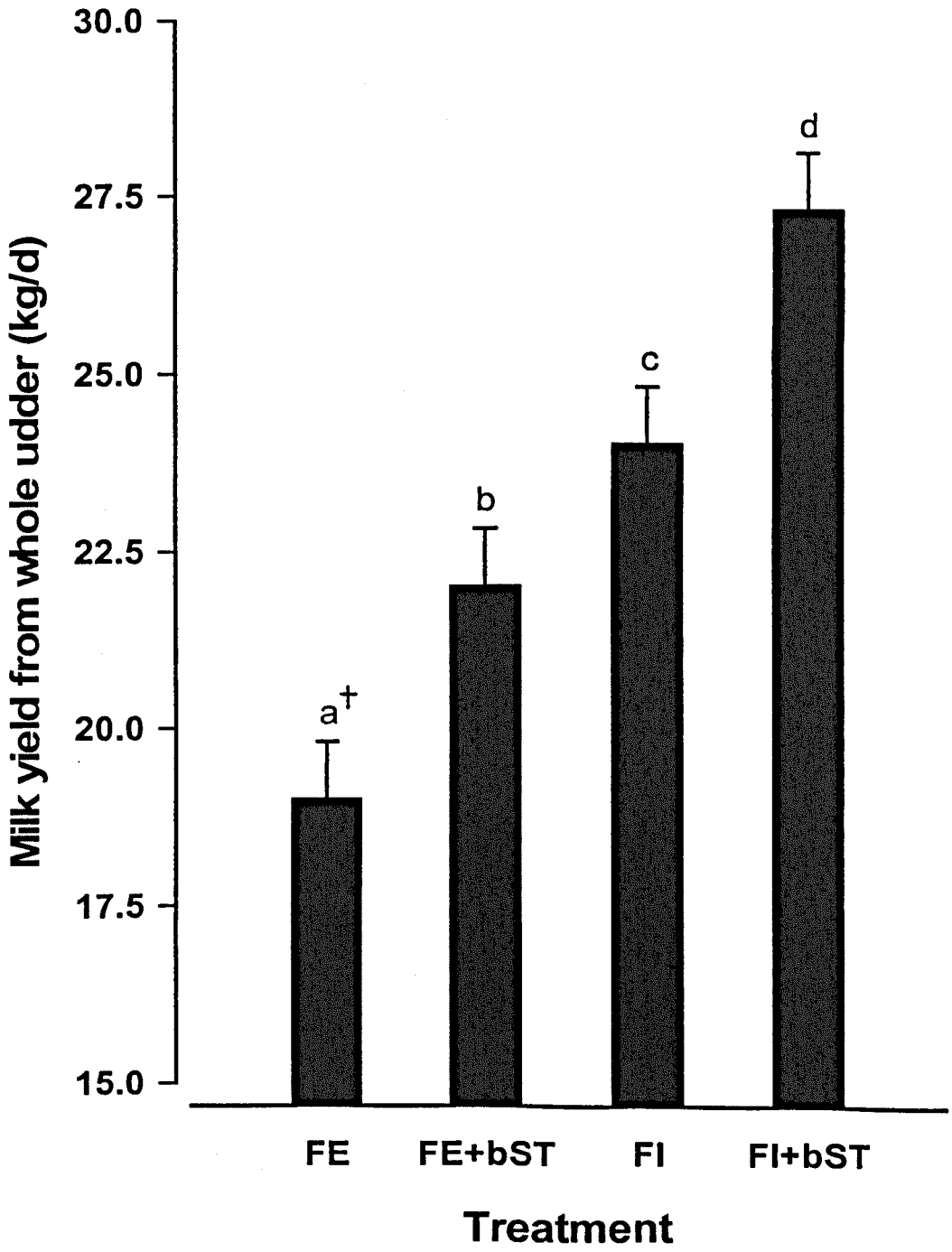


Figure 5.1 Milk yield from whole udder (kg/d) in Experiment 6 (Mean ± SED of 8 cows). †Unlike letters signify statistically significant difference ($P<0.05$) by *t*-test.

Table 5.4 Milk production and mammary blood flow (MBF) from whole udder, and body weight of cows in Experiment 6

	FE	FE+bST	FI	FI+bST	SED[1]	P value		
						Diet	bST	Diet x bST
DM intake, kg/d								
Silage	10.9	10.7	11.6	11.5	0.35	0.008	0.510	0.701
Total	18.0	18.1	19.9	20.1	0.34	<0.001	0.407	0.967
Milk yield, kg/d	19.0	22.0	24.4	27.3	0.81	<0.001	<0.001	0.961
Milk protein, g/kg	32.4	30.7	33.1	31.7	0.42	0.016	<0.001	0.631
g/d	616	675	803	865	23.2	<0.001	0.003	0.929
Milk fat, g/kg	42.7	47.1	39.2	41.6	1.75	0.003	0.015	0.445
g/d	806	1034	950	1137	52.0	0.005	<0.001	0.582
Milk lactose, g/kg	47.7	48.6	47.3	48.2	0.37	0.200	0.005	0.975
g/d	907	1070	1155	1317	41.3	<0.001	<0.001	0.986
MBF, l/min	15.1	16.8	13.8	15.2	1.05	0.064	0.052	0.826
Body weight, kg	589	591	600	601	4.9	0.008	0.595	0.981

[1] SED, standard error of differences. For comparisons between means use LSD = 2.145 (t value) x SED ($P<0.05$).

For the main effects, mammary blood flow tended to be increased by the feather meal diet and bST. However, no significant differences in mammary blood flow between means were found with the exception that mammary blood flow was significantly higher for the FE + bST treatment than for the FI treatment. Body weight was higher for the fish meal diet than for the feather meal diet.

Results for the effects of frequent milking on milk production and mammary blood flow are given in Table 5.5. As regards the main effects of frequent milking on milk production and mammary blood flow, 3x milking significantly increased yields of milk and milk lactose, fat and protein, and the concentration of lactose, but there were no significant differences in the concentrations of milk protein and fat between 2x and 3x milking. However, in a comparison of the effects of frequent milking within each treatment, 3x milking significantly increased milk yield in the FI and the FI + bST treatments but did not affect it in the FE and the FE + bST treatments. This is true also for yields of milk protein and lactose. The 3x milking increased milk fat yield in the FI treatment but did not affect it in the other treatments. There was a small, but significant, increase in the concentration of milk lactose in the FE and the FI + bST treatments in response to 3x milking. No significant differences were seen in mammary blood flow between 2x and 3x milking when comparisons were made within each treatment.

For the main effects, the concentration of haemoglobin in blood was significantly lower for the bST treatment than for the control (Table 5.6). When comparisons were made within each diet, the concentration of haemoglobin was significantly ($P<0.05$) reduced and tended ($P<0.10$) to be decreased by bST in the feather and the fish meal diets, respectively. The concentrations of NEFA tended ($P<0.10$) to be higher for the bST treatment than for the control. Although there was a significant interaction in the ratio of 3-methylhistidine/creatinine, no significant differences in the ratio of 3-methylhistidine

Table 5.5 Milk production and mammary blood flow (MBF) from each half-udder

	FE		FE+bST		FI		FI+bST			P value			
	2x	3x	2x	3x	2x	3x	2x	3x	SED[1]	Frequency	Frequency x Diet	Frequency x bST	Frequency x Diet x bST
Milk yield, kg/d	9.7	10.0	10.9	11.4	11.5	12.8	12.7	13.7	0.36	<0.001	0.021	0.865	0.385
Milk protein, g/kg	32.5	32.2	31.5	31.5	32.1	32.2	31.9	32.0	0.16	0.797	0.101	0.219	0.383
g/d	310	317	332	351	376	422	408	444	12.7	<0.001	0.019	0.946	0.316
Milk fat, g/kg	42.9	42.0	46.3	46.4	39.5	41.0	42.2	41.2	1.25	0.903	0.567	0.450	0.108
g/d	424	428	497	519	468	529	522	542	19.2	0.006	0.131	0.506	0.095
Milk lactose, g/kg	47.2	47.9	48.2	48.2	47.8	48.1	47.9	48.4	0.20	<0.001	0.900	0.171	0.020
g/d	465	483	529	551	547	612	605	659	17.6	<0.001	0.017	0.807	0.641
MBF, l/min	7.0	8.1	7.8	9.1	6.3	7.5	7.0	8.2	0.79	0.007	0.964	0.884	0.886

[1] SED, standard error of differences. For comparisons of the effects of frequent milking within each treatment use LSD = 2.056 (t value) x SED (P<0.05).

Table 5.6 The composition of plasma and blood, and the ratio (μmol/mmol) of 3-methylhistidine/creatinine in urine in Experiment 6

	FE	FE+bST	FI	FI+bST	SED[1]	P value		
						Diet	bST	Diet x bST
Glucose, mmol/l	3.47	3.64	3.54	3.63	0.157	0.807	0.262	0.718
Urea-N, mmol/l	2.82	2.86	3.25	2.79	0.361	0.503	0.411	0.347
Haemoglobin, g/l	87	80	89	83	3.0	0.269	0.010	0.840
Albumin, g/l	34	34	35	33	1.1	0.971	0.433	0.133
NEFA[2], mmol/l	0.03	0.06	0.03	0.05	0.015	0.647	0.057	0.366
3-methylhistidine/ creatinine ratio	15.1	13.7	21.6	23.9	1.08	<0.001	0.617	0.031

[1] SED, standard error of differences. For comparisons between means use LSD = 2.145 (t value) x SED ($P<0.05$).
[2] Non-esterified fatty acids.

/creatinine between the control and the bST treatment were seen within each dietary treatment. The ratio of 3-methylhistidine/creatinine in urine was significantly lower for the feather meal diet than for the fish meal diet.

The concentrations of plasma hormones are shown in Table 5.7. For the main effects, increases in the concentrations of growth hormone, IGF-I and insulin were seen with bST administration. When comparisons were made between means, neither the difference in the concentration of IGF-I between the FI and the FI + bST treatments nor that of insulin between the FE and the FE + bST treatments reached statistical significance.

The concentrations of plasma free amino acids are shown in Table 5.8. Compared with the fish meal diet, the feather meal diet produced significantly greater concentrations of total AA, non-essential AA, Val, Phe, Leu, Ser, Gly and Tyr, but lower concentrations of His, Met, Lys, Asn and Tau. Compared with the control, bST treatment was associated with lower concentrations of His, Arg, Trp, Leu, Lys and Orn, and higher levels of Thr and Ser. There were significant interactions in the concentrations of His, Ser, Tau and non-essential AA.

5.4 Discussion

Administration of bST resulted in increased milk yield for both the feather and the fish meal diets. Increases in milk energy output in response to bST were 13 and 11 MJ/d in the feather and the fish meal diets, respectively. However, in this short-term experiment, the increased milk yield with bST treatment was not accompanied by changes in feed intake for either dietary treatment. It is known that increase of food intake in response to bST occurs after a few weeks of bST treatment (Bauman, 1992; Bauman and Vernon, 1993). But the digestibility of the diet, the energy expenditure for

Table 5.7 The concentrations (ng/ml) of plasma hormones in Experiment 6

	FE	FE+bST	FI	FI+bST	SED[1]	P value		
						Diet	bST	Diet x bST
Growth hormone	3	110	3	118	16.8	0.740	<0.001	0.699
IGF-I	79	170	114	172	32.8	0.439	0.006	0.499
Insulin	0.22	0.28	0.19	0.32	0.041	0.698	0.005	0.218

[1] SED, standard error of differences. For comparisons between means use LSD = 2.145 (t value) x SED (P<0.05).

Table 5.8 The concentrations (μmol/l) of plasma free amino acids in Experiment 6

	FE	FE+bST	FI	FI+bST	SED[1]	P value		
						Diet	bST	Diet x bST
Histidine	7	6	11	8	0.8	<0.001	0.002	0.037
Threonine	222	238	225	241	9.9	0.658	0.038	0.976
Arginine	103	96	127	98	10.3	0.091	0.028	0.161
Tryptophan	42	38	45	41	2.5	0.077	0.041	0.945
Methionine	28	29	44	43	2.3	<0.001	0.805	0.541
Valine	355	348	278	245	28.8	<0.001	0.336	0.538
Phenylalanine	99	101	89	87	4.1	<0.001	0.992	0.488
Isoleucine	136	127	147	124	12.5	0.685	0.094	0.434
Leucine	140	133	130	101	12.0	0.026	0.047	0.216
Lysine	73	63	114	78	11.1	0.003	0.011	0.119
Aspartic acid	6	6	6	7	0.5	0.086	0.252	0.975
Glutamic acid	116	108	120	124	7.3	0.066	0.676	0.273
Serine	317	427	205	238	22.8	<0.001	<0.001	0.030
Glycine	1146	1216	1061	1040	41.9	<0.001	0.422	0.148
Alanine	338	353	368	336	18.8	0.628	0.527	0.103
Tyrosine	100	108	94	97	4.6	0.022	0.103	0.352
Asparagine	41	43	47	46	2.4	0.037	0.894	0.478
Taurine	30	31	37	32	1.8	0.011	0.194	0.026
Glutamine	104	99	107	109	8.7	0.304	0.762	0.574
Ornithine	23	19	29	21	3.0	0.079	0.011	0.280
Essential AA	1205	1179	1211	1066	77.3	0.342	0.139	0.297
Non-essential AA	2219	2412	2074	2048	65.0	<0.001	0.092	0.032
Total AA	3424	3591	3285	3113	126.8	0.004	0.978	0.080

[1] SED, standard error of differences. For comparisons between means use LSD = 2.145 (t value) x SED ($P<0.05$).

maintenance and the partial efficiency of milk synthesis are not altered. Thus, until the point at which food intake is increased, nutrients required for increased milk production with bST treatment are supplied by changing nutrient partitioning or mobilizing body reserves. Indeed, the numerically similar responses to bST with the two diets may reflect the short-term, change over design of the experiment.

Dietary effects on milk production largely agree with the results of experiments in previous chapters. Compared with the feather meal diet, the fish meal diet substantially increased yields of milk and milk constituents. The effects of amino acid supply on the response to more frequent milking also agree well with the results of experiments in Chapter 4. Thrice-daily milking increased milk yield for the fish meal diet but did not affect it for the feather meal diet. It has been shown that the systemic action of bST and the local galactopoietic action of frequent milking are additive in increasing milk yield in dairy cows (Knight *et al.*, 1992; Speicher *et al.*, 1994). Results for the fish meal diet supported this view, with increased milk yield in response to frequent milking with bST administration. But, for the feather meal diet, little response to frequent milking with bST administration lend this no support. It is clear that the supply of nutrients, particularly amino acids was limiting on the feather meal diet since milk yield was lower even for the feather meal with bST administration than the fish meal diet alone. Although bST increased milk yield for the feather meal diet, the absence of a significant increase in milk yield in response to frequent milking suggests that frequent milking is, at best, a weak stimulus to changing nutrient partitioning.

Yields of milk protein did not increase in proportion to the increases in milk yield so milk protein concentrations were reduced in response to bST for both diets. In the present study, the supply of metabolizable protein was substantially in excess of requirement by,

at least, 35 % for both diets and this does not support a general view that, in cows in positive nitrogen balance, bST does not affect milk protein concentration (Peel *et al.*, 1983; Sechen *et al.*, 1989; Vicini *et al.*, 1991). Since little or no increases in feed intake were seen in response to bST for both diets, it would seem likely that the decreased milk protein content is linked to the fall in energy status of the cows. It has been suggested, from an analysis of a large number of feeding trials, that there is a positive relationship between energy status of the cow and milk protein content (Coulon and Rémond, 1991).

When bST-treated cows are in positive energy balance, milk fat content is not altered. However, when cows are in negative energy balance, milk fat content increases with bST treatment so that an increase in milk fat yield is greater than milk yield (Peel and Bauman, 1987; Tyrrell *et al.*, 1988; Bauman and Vernon, 1993). In the present study, the supply of metabolizable energy (ME) was in substantial excess of requirement by, at least, 15 % in all treatments. But, relative to the other treatments, the FE + bST treatment significantly increased the concentration of milk fat. Whether bST enhances the effect of dietary amino acid imbalance on increased milk fat synthesis is not known. However, it is worth noting that both bST (Mepham *et al.*, 1984; Davis and Collier, 1985) and deficiency in the supply of histidine (Bequette *et al.*, 2000) have been shown to increase mammary blood flow. That the mammary uptake of fat precursors seems to be regulated primarily by their arterial concentrations (Miller *et al.*, 1991; Nielsen and Jakobsen, 1994) suggests that the increased mammary blood flow might lead inevitably to an increased uptake of fat precursors by the gland (Cant *et al.*, 1999). In the present study, a statistically significant difference in mammary blood flow was not seen for bST or for the feather meal diet alone. However, as mentioned in Chapter 4, with respect to the sensitivity of technique, it would seem unlikely that 10 % increase in mammary blood flow in response to bST or the feather meal diet alone would be sufficient to reach

statistical significance. However, when these two factors were combined, a significant increase (22 %) in mammary blood flow for the feather meal with bST administration was seen.

For both dietary treatments, no significant differences in the concentration of plasma NEFA and the ratio of 3-methylhistidine/creatinine in urine between the control and the bST treatment were seen, and this suggests that increases in nutrient supply to fuel increased milk yield in response to bST could have derived from changing nutrient partitioning between the body and the mammary glands rather than from mobilizing body reserves. The excess ME intake is sufficiently large to allow such an effect.

As the accumulation or mobilization of muscle protein is determined by the relative contributions of protein synthesis and breakdown (i.e. protein turnover), changes in the rate of protein turnover affect the excretion of 3-methylhistidine. For example, in growing steers, rapid growth is accompanied by a high rate of myofibrillar protein degradation so increasing the urinary output of 3-methylhistidine (Gopinath and Kitts, 1984). In studies of nutritional effects on protein metabolism in rats, protein deficiency has been shown to be associated with a decrease in the rate of muscle protein breakdown (Haverberg *et al.*, 1975; Funabiki *et al.*, 1976). But, protein repletion was accompanied by an increased rate of muscle protein turnover. In the present study, the intention was only to use the determination of 3-methylhistidine as an index of changes in body protein degradation in response to bST within a dietary treatment. The higher 3-methylhistidine/creatinine ratio for the fish meal diet presumably reflects differences in protein turnover on the two diets.

CHAPTER SIX

GENERAL DISCUSSION

6.1 Nutrition and mammary function

The overall aim of the thesis was to examine the influence of nutrition (specifically protein and amino acid nutrition) on the response of lactating dairy cows to an increased frequency of milking. As a starting point, the dietary treatments chosen were evaluated in a long-term experiment (Chapter 3), to determine the effect of the two treatments on milk production and mammary function. Key enzyme activities regulating milk synthesis and total DNA in the mammary gland were determined as indicators of mammary function. Although the feather meal diet markedly decreased milk yield by around 21 % relative to the fish meal diet, little difference in mammary function between the feather meal and the fish meal diets was detected. Statistically significant effects on FAS and PCNA that occurred at isolated time points were difficult to interpret because there were differences between the two groups of cows when both consumed the control diet in Experiment 1. The fact that the two treatments supported levels of milk production that differed by around 21 % and yet no clear differences were detected in the measurements of mammary function suggests either that the measurements made are not sensitive indicators of mammary function or, alternatively, metabolic capacity of the mammary gland was in substantial excess of that required on the feather meal treatment. There is some support for the latter suggestion in that cows eating the feather meal diet respond to intravenous infusion of amino acids by markedly increasing milk secretion within 24 h (Kim *et al.*, 1999; 2000). Such a rapid response would seem incompatible with a change in mammary function.

In view of these results, it was decided not to include measurements of mammary function in the experiments that followed. This decision was taken on both scientific and practical grounds. Scientifically, the evidence from the first two experiments suggested that the measurements were unlikely to be central to the interpretation of the results; practically, recovery from biopsy took around 2 weeks, which would have seriously affected the interpretation of the results of short-term experiments of the type planned for Chapters 4 and 5.

Although it has been suggested that a gradual decrease in the number of mammary epithelial cells within the mammary glands largely accounts for the decline in milk production with advancing lactation (Capuco *et al.*, 2001), the effect of nutrition on mammary function in lactating dairy cows has received little attention. On the other hand, factors other than nutrition have been studied in goats and in cows. In goats, thrice-daily milking increased the number of mammary cells in the long term (around 37 weeks) (Henderson *et al.*, 1985; Wilde *et al.*, 1987a) and key enzyme activities regulating milk synthesis in the short term (2 weeks) (Wilde *et al.*, 1987a). However, in lactating cows milked four times daily for 4 weeks, enzyme activities in the mammary gland are not significantly affected (Hillerton *et al.*, 1990; Knight *et al.*, 1992) and little information is available on changes in mammary cell number in response to increasing milking frequency. It is not known whether these apparent differences between cows and goats in the response of enzyme activities to increasing milking frequency are due to species differences or to different nutritional treatments. This remains to be investigated. Nonetheless, the longest period adopted in the present studies for thrice-daily milking with the fish meal diet was 2 weeks and thus, it would seem unlikely that changes in mammary enzyme activities would occur in that time in response to thrice-daily milking.

There are inconsistent reports of changes in mammary function in response to bST. Binelli *et al.* (1995) reported that administration of bST to lactating cows for 9 weeks increased total RNA in the mammary gland but did not increase total DNA. In contrast, in lactating cows, Capuco *et al.* (2001) showed that injection of bST for 1 week increased the rate of cell proliferation in the mammary gland. Beswick and Kennelly (1998) reported that mRNA and protein abundance of acetyl-CoA carboxylase and fatty acid synthase was not affected by bST.

6.2 Effects of milking frequency on milk secretion

Local control of milk secretion is thought to underlie the responses to changes in the frequency of milking. In goats, when one of the two glands was milked hourly, the rate of milk secretion increased only in that gland (Linzell and Peaker, 1971). Thus, it was proposed that some form of local chemical inhibition was operating within each mammary gland. Thereafter, experimental evidence accumulated, supporting the hypothesis of a chemical inhibitor of milk secretion present in milk. For example, when an inert, isotonic solution, equal in volume to the milk removed at one of the three milkings, was infused into the lumen of one gland, the rate of milk secretion still increased, showing that the composition of milk, rather than its physical presence, was the key determinant in local control of milk secretion (Henderson and Peaker, 1984). It has been shown that a milk fraction containing whey proteins, named feed back inhibitor of lactation (FIL), can inhibit milk secretion (Wilde *et al*, 1987). Moreover, direct infusion of FIL into one gland of lactating goats produced a temporary dose-dependent reduction in milk yield only in that gland (Wilde *et al.*, 1988). Consequently, an increase in milk yield in response to increasing milking frequency is ascribed to more frequent removal of FIL from the gland. Besides FIL, a plasmin-induced β-casein breakdown product

(fraction 1-28), a potent blocker of potassium channels in the apical membrane of the mammary epithelium, has been reported to inhibit milk secretion (Silanikove *et al.*, 2000).

The opposite responses can be expected when milking frequency is decreased from twice to once daily. However, the effect of once-daily milking on decreased milk secretion may not be wholly explained by FIL. Other factors may play a role. Notably, once-daily milking was associated with increases of tight junction permeability in the mammary gland, decreases in mammary blood flow and changes in cell shape due to the alveolar distension (Davis *et al.*, 1999). As mentioned above, however, experimental evidence suggests that the degree of alveolar distension is not responsible for an increase in milk yield in response to thrice-daily milking. Little information is available on the response of tight junction permeability and mammary blood flow to thrice-daily milking. However, the ratio of Na to K in milk, an indicator of tight junction permeability, has recently been reported to be lower for thrice-daily milking than for twice-daily milking, indicating that tight junctions became tighter in response to thrice-daily milking (Sorensen *et al.*, 2001). There are inconsistent reports of changes in mammary blood flow to increasing milking frequency. In contrast to the findings of an earlier study (Maltz *et al.*, 1984), Bequette and Backwell (1997) reported that, during unilateral frequent milking of goats, subsequent increases in milk and protein yields were mirrored by increases in mammary blood flow.

Whatever factors might be involved in the control of milk secretion in response to increasing or decreasing milking frequency, it is noteworthy that injection of bST almost negated the loss of milk yield in response to once-daily milking (Davis *et al.*, 1999), suggesting that the mammary gland was operating below its maximum capacity. Furthermore, the marked increases in milk yield within 24 h in response to intravenous

infusions of the limiting amino acid (Kim *et al.*, 1999; 2000) suggest that nutrient supply might override the effects of local inhibition of milk secretion by FIL. Again, reducing feed intake prevented any increase in milk secretion in response to hourly milking (Blatchford and Peaker, 1983). Indeed, it has been pointed out that, since the stimulatory response to hourly milking is mediated locally, any systemic rate limitation by substrate supply acting directly on the mammary gland would prevent the response (Blatchford and Peaker, 1982).

6.3 Nutrition and the response of the mammary gland to increased frequency of milking

Results for the series of experiments in Chapters 4 and 5 with cows given the feather meal diet show that even though dietary ME is in considerable excess, a deficiency of specific amino acids (His, Met, Lys) can prevent any increase in milk yield in response to increasing the frequency of milking from twice to thrice daily. In contrast, when cows consumed a similar level of excess ME and a similar level of rumen-undegradable protein where the protein was of better amino acid balance (fish meal), the increased frequency of milking led to increased milk yield.

When the responses of milk secretion to increased milking frequency on the feather meal diet are examined, they show that, although secretion of protein is unchanged and lactose changes little, secretion of fat responds differently between experiments. Thrice-daily milking increased the secretion of milk fat in Experiments 3 and 4 but did not affect it in Experiments 5 and 6. One possible explanation would be that the level of excess ME would result in different responses in milk fat secretion. However, responses seem contrary to expectation in that the greater excess of ME in Experiments 5 and 6 was associated with no response to thrice-daily milking.

Compensatory effects (increase in secretion of the test gland, at the expense of the control), which have been reported before (Dewhurst and Knight, 1994; Stelwagen and Knight, 1997; Sorensen and Knight, 1999), might be expected to be particularly evident when the supply of nutrients is deficient. In cows, when milking frequency was increased from two to four times daily with subsequent treatments of bST and thyroxine, milk yield was increased progressively with each treatment applied such that the highest milk yield was obtained with the complete combination of frequent milking, bST and thyroxine. But a further increase of milk yield was seen in the half-udder that continued on four times daily milking when the frequency of milking in the other half was reduced from four times to twice daily. Although data for feed intake were not given, it would seem unlikely that, within such a short time span (5 d with each treatment), increases of feed intake would occur in response to those treatments. It might be expected that, in these cows, nutrients for milk secretion would be in relatively short supply. It would then follow that decreasing milking frequency and the associated reduction in milk secretion in that half of the udder might provide additional nutrients to the other udder half. In contrast, it might be expected that, when nutrients are deficient, increasing the frequency of milking in one half udder might reduce nutrient supply to the other udder half. As mentioned in Experiment 4, in which cows were given the feather meal diet, it would seem that a small, but significant decrease in milk yield from the glands milked twice daily contributed to the overall response to thrice-daily milking, when measured as the difference between treatment and control glands.

As the compensatory responses to increasing or decreasing milking frequency occur very quickly, it seems unlikely that developmental changes in the mammary gland are responsible because increases of enzyme activity of mammary epithelial cells are not evident until after 4 weeks of treatment (Knight *et al.*, 1992). Another possible

explanation would be that changes in the concentrations of arterial nutrients and mammary blood flow led to an increased the supply of nutrients to the gland. As mentioned above, the effects of frequent milking on mammary blood flow are unclear. In Experiment 4 where the compensatory response was examined, changes in mammary blood flow in response to thrice-daily milking were not evident. As discussed in Chapter 4, however, when changes in milk yield in response to thrice-daily milking are small, it is doubtful whether any associated changes in mammary blood flow (assuming they were roughly proportionate to the increase in milk secretion) would be great enough to be detected by the technique used here. It should be remembered, however, that the proportion of capillaries perfused with blood and the regulation of capillary permeability might play an important role in delivering nutrients to the mammary gland (Prosser *et al.*, 1996) and that these effects could conceivably operate independently of effects on total mammary blood flow. As the concentrations of arterial nutrients were not measured, no definite statement can be made, but if arterial concentrations were increased in response to the increased frequency of milking, then an increased supply of nutrients to the control half-udder would be expected. Again, when nutrients required for milk synthesis are in excess, the repartitioning of blood flow between the udder-halves might still occur but compensation would not be evident in milk secretion because the control half of the gland might still be presented with more nutrients than it needed. Another possibility worth mentioning is that mammary blood flow to the test half might be increased only in conditions of nutrient deficiency. Under an imposed limitation on histidine supply for milk production, mammary blood flow increased, presumably in an attempt to overcome the limitation of histidine supply to the udder (Bequette *et al.*, 2000). A similar tendency was also observed during an experimentally imposed deficiency of leucine (Bequette *et al.*, 1996). Nutrient deficiency, particularly of amino acids, might enhance the effect of

increased milking frequency on mammary blood flow. If that were the case, it could be said that the physiology of the response to increasing the frequency of milking would be determined by nutrient supply. Clearly, a number of factors, nutrient supply prominent among them, probably interact in determining the physiological response of the gland to an increased milking frequency, which might explain the apparent contradictions in the literature.

6.4 Milking frequency and the partitioning of nutrients between body and udder

Although an increase in the secretion of milk protein in response to thrice-daily milking was blocked on the feather meal diet, presumably by the specific amino acid deficiencies mentioned above, the yield of milk protein with this diet was markedly increased by around 10 % when the cows were injected with growth hormone. This means that the amino acids necessary to fuel an increase in secretion of milk protein were potentially available in cows eating the feather meal diet. Under the action of growth hormone, a potent repartitioning agent, extra amino acids were made available to the mammary gland or, more precisely, extra amino acids were synthesized into milk protein. Moreover, as discussed in Chapter 5, the increased energy output in milk that was induced by injection of growth hormone could be accounted for by a repartitioning of ME intake, without the need for any mobilization of tissue. Again, the lack of effect on 3-methylhistidine excretion would suggest that amino acids were diverted to the mammary gland without effects on the degradation rate of muscle protein. This does not rule out mobilization of muscle protein because it is possible that the rate of muscle protein synthesis was reduced (Funabiki *et al.*, 1976), leading to net mobilization with no change in release of 3-methylhistidine from the muscle. In addition, of course, mobilization of body proteins other than muscle, e.g. skin, bone, etc. could be playing an important part

in the response. Indeed, the fractional synthesis rates of tissue proteins including those of skin, head and feet are markedly decreased in early lactation (Champredon *et al.*, 1990; Baracos *et al.*, 1991) when the demand for amino acids for milk secretion is especially high.

It has been calculated, using a depletion/repletion technique, that labile protein reserves in the cow may be as much as 0.25 of total body protein (Botts *et al.*, 1979). The transition from the dry period to early lactation involves a marked decline in the rate of protein synthesis in muscle, the largest protein store in the body (around 0.60 of whole body protein), while the mammary gland shows a substantial increase in the rate of protein synthesis (Champredon *et al.*, 1990). Thus, the decreased rate of protein synthesis in muscle, skin and possibly other tissues would permit diversion of amino acids towards the mammary gland in early lactation. Since it has been suggested that metabolic adaptations leading to the galactopoietic response of bST-treated cows are similar to those that support increasing milk yield during early lactation (Burton *et al.*, 1994), the amino acids needed to fuel the increases in milk protein yield in response to bST might arise from the reduced rate of protein synthesis in non-mammary tissues. However, it may not be that simple because, in growing animals, bST is known to increase protein accretion in muscle (Nieto and Lobley, 1999). This apparent contradiction between growing animals and lactating cows in response to bST has been mentioned by Bell (1995) who suggested that, in the mature cow, the capacity for anabolic responses to bST in skeletal muscle might be small relative to that in the mammary gland during lactation. Indeed, experimental evidence suggests that the relative responsiveness of skeletal muscle changes with stage of lactation. Chilliard *et al.* (1991) reported that injection of bST during early to mid lactation did not affect body protein reserve but increased it in late lactation. Although it is known that the coordination of nutrient partitioning in the

lactating cow entails interactions between nutrient supply and hormonal regulation, the mechanisms underlying the associated changes in tissue protein metabolism, especially that of muscle in response to repartitioning agents, are unclear. With the level of experimentation in this thesis, it is not possible to identify precise changes in metabolism of amino acids induced by injection of growth hormone. That would require the use of sophisticated labelling techniques for the various pools of amino acids and proteins in the body. In the absence of such quantitative information, the present experiments can be interpreted only speculatively.

No matter what the precise mechanisms may be, it is clear that any repartitioning achieved by injection of growth hormone on the feather meal treatment, could not be achieved by increasing milking frequency alone. Thrice-daily milking could not influence amino acid partitioning to any measurable extent. However, experiments reported in the literature (DePeters *et al.*, 1985; Kazmer *et al.*, 1986), and the results of the present experiments with the fish meal diet, show that thrice-daily milking can increase partition of nutrients to the udder at the expense of body tissue (since, by definition, any increase in milk secretion in the absence of a change in feed intake must signify a change in nutrient partition). Indeed, with the fish meal diet, responses of milk production to growth hormone and to milking more frequently were additive, confirming earlier reports (Knight *et al.*, 1992; Speicher *et al.*, 1994). This additivity might reflect two modes of action, or it might signify an increased intensity of the same repartitioning stimulus. Since it can be argued that injection of growth hormone produces increases in plasma levels of growth hormone that are well beyond the normal physiological range, it would seem unlikely that increasing the frequency of milking could enhance the effect of growth hormone itself on nutrient partitioning. It would then seem more likely that the two treatments affect nutrient partitioning in the same general direction but by different mechanisms.

The limited results in the literature on changes of feed intake in response to thrice-daily milking suggest that the response of feed intake to thrice-daily milking and to bST may be different. It is well known that increases of food intake in response to bST occur after 6 - 8 weeks of treatment (Chilliard, 1992). Therefore, until the point at which food intake is increased, nutrients required for increased milk production must be supplied by changing nutrient partitioning or mobilizing body reserves. On the other hand, increases in milk yield in response to thrice-daily milking are accompanied by no effect on feed intake (Pearson *et al.*, 1979; DePeters *et al.*, 1985; Barnes *et al.*, 1990) or by very small increases (Poole, 1982; Amos *et al.*, 1985). However, milking more frequently than three times a day may enhance the effect on feed intake. When the frequency of milking was increased from three times to six times daily during the first 6 weeks of lactation, feed intake was increased by the six times-daily milking (Bar-Peled *et al.*, 1995). But it is surprising that feed intake could be increased during that time when cows are normally in negative energy balance because of an inability to increase feed intake. Clearly, this is an area needing further investigation. Nevertheless, since the shortest period adopted in those thrice-daily milking experiments was 20 weeks, the lack of response of feed intake to the thrice-daily milking treatment cannot be related to the length of experimental period. This means that, in the long term, if the magnitude of the milk yield response to thrice-daily milking and to bST were to be similar, the efficiency of use of nutrients for milk production would be higher for thrice-daily milking than for the bST treatment because of more favourable partition of nutrients to the udder.

Changes in body tissue metabolism in response to increased frequency of milking have not been detailed. Although the results of experiments cited above indicate that such changes can occur (because nutrient partitioning must have been affected) the results in Chapter 5 further indicate that such effects depend on amino acid nutrition. This contrasts

with the effect of bST on nutrient partitioning, which was not affected by amino acid nutrition.

In general, the effect of bST on increasing milk production during early lactation is small relative to that in mid to late lactation since cows are more likely to be nutrient deficient in early lactation (Bauman and Vernon, 1993). But, even in nutrient deficiency, bST increases the concentration and yield of milk fat since bST increases lipolysis or inhibits lipogenesis in adipose tissue, depending on the cow's energy status. When the results of DePeters *et al.* (1985) are examined, heifers that were near zero energy balance, based on an efficiency of utilization of ME for lactation of 0.62 (Agricultural Research Council, 1980), showed little response of milk production to thrice-daily milking (DePeters *et al.*, 1985). Similarly, when high-yielding cows were milked thrice daily, lack of increases in feed intake prevented any increase of milk yield in response to thrice-daily milking (Barnes *et al.*, 1990). In these experiments, milk fat concentration was slightly decreased rather than being increased in response to thrice-daily milking, suggesting that, unlike bST, frequency of milking might have little effect on adipose tissue. This observation, together with the results from Experiments 3, 4, 5 and 6 suggests that the response to thrice-daily milking might be achieved only when nutrient supply is in excess of requirement, although it should be noted that results of Experiment 5 show that extra ME added to an ME supply is already in excess led to no further increase in the response to thrice-daily milking.

6.5 Future work

Future work should consider carefully the choice of experimental technique, which, in turn, would depend on the questions being asked. The experimental approach would

depend, in particular, on whether the aim was to uncover underlying physiological mechanisms or whether possible application in practice was of more immediate concern.

6.5.1 Short-term versus long-term experiments and the use of whole-udder and half-udder milking

It is difficult to see how the effects of growth hormone injection on the feather meal treatment could be sustained in the longer term. Indeed, the results from short-term changeover experiments must be interpreted carefully. For example, if the responses to growth hormone on the feather meal treatment depend on mobilization of body protein, then the combination of short period lengths and a changeover design, leading to alternating depletion and repletion of body protein stores, could give a very misleading impression of responses to be expected under practical conditions. Again, responses to bST in short-term trials will be determined by nutritional status and whether feed intake is increased. As well as influencing the magnitude of the response of milk yield, such factors will affect the changes in milk composition, particularly milk protein concentration. As would be expected, if bST induces repartition of nutrients towards the mammary gland at the expense of body, and feed intake is not increased, the fall in energy status of the cow will be reflected in a fall in the concentration of milk protein as seen in Experiment 6 for both the feather meal and fish meal treatments.

Similar general concerns surround the choice of whole-udder versus half-udder milking. Particularly when nutrient supply is limiting, but perhaps more generally, the use of half-udder milking may exaggerate the relative response to increased frequency of milking. Again, if the aim is to investigate the effects of increased milking frequency on nutrient partitioning, applying the treatment to only one half of the gland may provide

insufficient stimulus. On the other hand, half-udder milking is essential if questions such as compensatory changes in milk secretion and blood flow are to be addressed.

6.6 Overall implications

If we could alter the shape of the lactation curve, it would allow fundamental changes to dairy practice. An example would be the economic use of extended lactations, with improved welfare of cows, reduced production of unwanted calves, and possible benefits for milk processing (Knight, 1998).

In considering the lactation curve, a fundamental question is whether the decline in milk yield, from peak to drying-off, is primarily due to a programmed fall in metabolic capacity of the mammary gland, or to a fall in the supply of nutrients to the gland brought about by a programmed change of nutrient partitioning in favour of other tissues. Overall, results in this thesis support the view that the mammary gland itself is operating below its metabolic capacity, in the sense that it was able to increase milk secretion rapidly in response to an increase in nutrient supply, to an increase in milking frequency (provided the supply of amino acids was adequate), or to injection of the cow with growth hormone. Moreover, the measurements of mammary function in the experiments lend no support to a limitation of metabolic capacity. To confirm unequivocally that the mammary gland retains excess capacity in late lactation is more difficult. As lactation advances, the marginal response of milk secretion to an increase of ME intake can be very low– even in mid–lactation it can be as low as 0.25 (Friggens *et al.*, 1995). This means that, within practical constraints, it is difficult to obtain a measurable increase of milk secretion in response to an increase in dietary intake of ME. However, the *primary* cause of the low response is not known–capacity of the gland or nutrient partition? What is needed is to determine the response to the direct intravascular infusion of complete mixtures of

nutrients into the mammary gland. Attention should be given to overcoming the technical difficulties associated with experiments of this type because the information they yield would allow us to identify the most useful approach to manipulating the persistency of lactation in the dairy cow.

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