NITROGEN METABOLISM OF THE DAIRY COW IN RELATION TO VOLUNTARY FEED INTAKE AND MILK PRODUCTION ON GRASS SILAGE DIETS

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by

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To my Parents

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SUMMARY

1. Experiments were conducted to investigate nitrogen metabolism of the dairy cow in relation to the control of the voluntary intake of grass silage and its utilization for milk production. Two aspects were considered: the effects of ammonia absorption from the rumen and the effects of changes in the post-ruminal supply of proteins and amino acids. In all experiments, dairy cows were given *ad libitum* access to grass silage and a fixed level of supplement based on barley and, where necessary, soyabean meal to give a total dietary CP concentration of 140-150 g/kg DM.

2. In the first study, effects on silage intake of the intraruminal infusion of urea in progressively increasing doses were investigated in four experiments. When urea was infused continuously, silage intake was depressed ($P<0.05$) when the total supply of N exceeded the equivalent of 250g CP/kg DM in the total diet. However, when the urea load was administered twice daily, as opposed to continuously, intake depression ($P<0.05$) occurred at the equivalent of 170g CP/kg DM. At the highest doses of urea, concentrations of NH_3 in peripheral blood increased and were accompanied by increased concentrations of glucose and reduced levels of insulin in plasma. It was concluded that the voluntary intake of high-protein silages may be depressed by factors associated with high rates of absorption of NH_3 from the rumen.

3. The effects of varying the amount and type of protein entering the abomasum were investigated in a series of four experiments. The first experiment examined responses to proteins differing in amino acid composition (casein and soya protein isolate (SPI) given as abomasal infusions and a fishmeal-based product (AP) given as a dietary supplement). All three treatments increased ($P<0.05$) the yields of milk (kg/d) and milk protein (g/d) relative to the basal treatment, the values being 16.9, 535; 18.8, 610; 19.5, 625; and 18.5 kg/d and 582 g/d for the basal, AP, casein and SPI treatment respectively. Responses to casein were greater ($P<0.05$) than to SPI.

In the second experiment the cows received abomasal infusions of casein or yeast protein

given in untreated (UTY) or heat treated (HTY) forms, each infusate supplying 200g CP/d. Relative to the basal treatment, casein increased (at least $P<0.05$) the yield of milk and milk constituents but the yeast products were without effect.

The third experiment examined responses to casein and to SPI in more detail. Each protein source was given in three levels of infusion supplying 100, 200 and 300g CP/d. For the highest dose of casein, milk yield was increased by 3.5 kg/d and the yield of protein by 37% over the basal treatment; corresponding values for the highest dose of SPI were 1.6 kg/d and 13% respectively. Responses of protein yield to casein infusion were close to linear but with SPI there was no response beyond the first dose level. It was calculated that casein infusion had a marked effect on the partition of energy utilization between body tissues and the mammary gland in favour of the latter.

A further experiment compared responses of silage intake and milk production to abomasal infusion of casein and an enzymic hydrolysate of casein containing about 40% of its nitrogen in the form of peptides. The yields of milk protein were identical for both treatments but the hydrolysate increased ($P<0.01$) the concentration and yield of milk fat compared with the casein treatment.

4. The final study comprised two experiments. The first experiment examined the effects of supplementing SPI with amino acids to make it equivalent to casein with respect to methionine and tryptophan (SPI+MT) or to total essential amino acids (SPI+AA). The five treatments were: basal diet; basal diet plus casein; basal diet plus SPI; basal diet plus SPI+MT and basal diet plus SPI+AA. All treatments were given as continuous abomasal infusions and supplied approximately 184g amino acid/d. All infusions increased ($P<0.05$) the yields of milk and milk protein relative to the basal treatment, the values being 20.2, 22.4, 21.5, 21.9 and 21.7 kg/d for milk yield and 626, 725, 673, 689 and 691 g/d for protein yield for the basal, casein, SPI, SPI+MT and SPI+AA treatment respectively. Casein produced a higher ($P<0.05$) yield than all other treatments.

Responses to abomasal infusion of casein or a hydrolysate of casein containing a small amount (about 15% of N) of peptides (PFCH) were compared in the second experiment.

Three dose levels of casein and of PFCH were used, equivalent to 100, 200 and 400 g/d of casein on a total amino acid basis. Casein induced greater responses in milk protein yield than PFCH: increases of milk protein output as a percentage of the basal treatment were 4, 14 and 18% for 100, 200 and 400 g/d dose of casein and 1, 3 and 14% for corresponding doses of PFCH.

5. It was concluded that the nutritional value of protein entering the post-ruminal gut of the dairy cow cannot be assessed solely in terms of its amino acid composition or absorbability. Other factors need to be considered. Among these are (1) the rate of release of amino acids and peptides during digestion and the effects of this on the rate of transfer of amino acids across the gut, (2) the release of peptides with potential biological activity.

CHAPTER 1

CHAPTER 1 INTRODUCTION

The process of ensiling is an age-old method of forage conservation, but in the last 20 years an intensification of animal production has been associated with a dramatic increase in the proportion of grass conserved as silage. Our understanding of the fermentation processes has improved, but although grass silage containing high concentrations of metabolizable energy and crude protein can be produced, there are limitations to its nutritional value. The low voluntary intake of grass silage is further compounded by an inefficient use of energy, together with a low and possibly unbalanced supply of amino acids. Furthermore, it is clear that silage is an extremely variable commodity and whilst it is recognized that intake and nutritional value may vary not only with the chemical composition but also with the characteristics of the silage fermentation, the relationships involved remain poorly characterized.

In Chapter 1 of this thesis the literature relating to the limitations to the nutritional value of silage, particularly those concerned with the supply of protein and/or amino acids are reviewed as an introduction to a series of investigations of relationships between nitrogen metabolism and voluntary intake and milk production in cows given silage-based diets.

1.1 Silage

Silage is defined as a forage conserved under anaerobic conditions in which naturally occurring microbes ferment plant carbohydrates to organic acids and as a result reduce pH and inhibit further fermentation. Considering the nutritional aspects, the objective of silage making is to achieve preservation while minimizing losses of nutrients and avoiding adverse changes in the chemical composition.

As well as events in the silo, silage composition depends on the composition of the crop and on processes occurring immediately post-harvest. As the crop matures, its digestibility and consequently its metabolizable energy (ME) value are reduced. Further, crude protein (CP) content decreases and, while soluble proteins of a relatively constant amino acid composition

continue to make up 0.75 to 0.85 of the CP (Lyttleton, 1973), the susceptibility of those proteins to attack by proteases may increase (Chamberlain and Thomas, 1984).

Following the cutting of grass, water soluble carbohydrates (WSC) are consumed by the plant's continuing respiration, but the changes in WSC content may be relatively small since the oxidized soluble sugars may be compensated by sugars released from the hydrolysis of polysaccharide (Carpintero, Henderson and McDonald, 1979). Substantial changes also occur in the nitrogen fraction where proteolysis and deamination increase the proportions of non-protein N, amino N, amide N and ammonia-N (Brady, 1960; Anderson, 1983). Further, there are modifications in the amino acid composition of the herbage. The concentrations of the majority of amino acids are reduced while the concentrations of proline, glutamine and asparagine are increased (Kemble and MacPherson, 1954).

1.1.1 Silage Fermentation

In the initial period after the crop has been placed in the silo, the aerobic metabolism of soluble carbohydrates continues. However, provided the silo is satisfactorily compacted and sealed to exclude air, anaerobiosis is achieved within hours and homolactic and heterolactic bacteria begin to proliferate with the relative exclusion of yeasts, Clostridia and fungi which are their potential competitors for the available sugars. The lactic acid bacteria metabolize hexose and pentose sugars to organic acids and will also metabolize the plant organic acids such as citrate, malate and oxaloacetae to lactate, acetate and 2,3-butanediol (Figure 1.1), and undertake partial breakdown (deamination or decarboxylation) of certain amino acids. Thus loss of amino groups with liberation of ammonia may lead to the formation of citrulline and ornithine from arginine, glutamate from glutamine, aspartate from asparagine and pyruvate from serine. Correspondingly, decarboxylation reactions, involving a loss of carbon dioxide, yield amines: tyramine from tyrosine, histamine from histidine, cadaverine from lysine and putrescine from ornithine. The lactic acid bacteria however have limited capacities for deamination and decarboxylation of amino acids and therefore ammonia and amines are produced in only small amounts in well preserved silages.

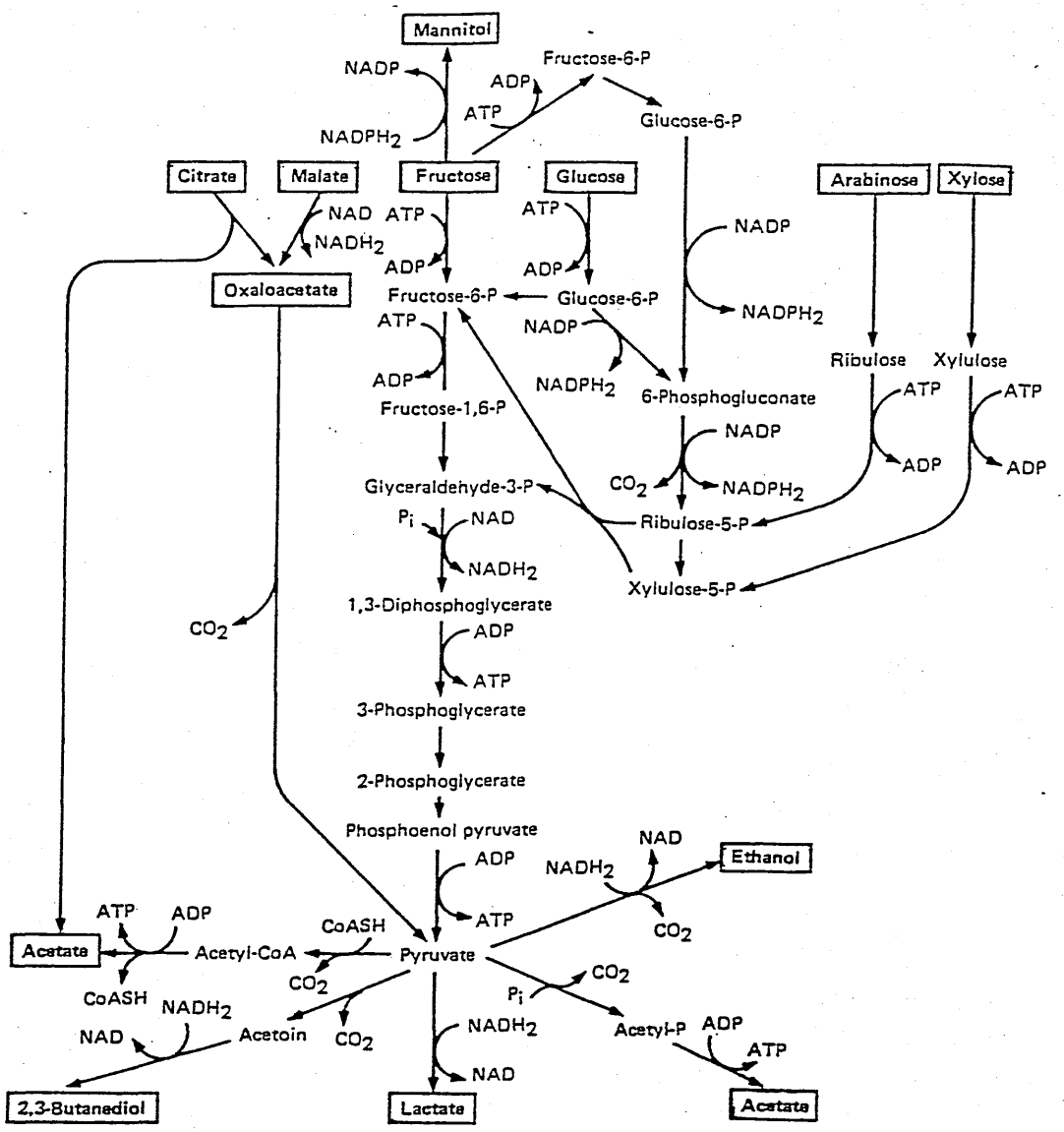


Figure 1.1 A schematic summary of pathways of fermentation for the homolactic and heterolactic bacteria. (After Thomas and Morrison, 1982)

Under adverse ensilage conditions, where the exclusion of air from the silo is inadequate or where wet, highly buffered crops of low sugar contents slow the initial fall in pH, Clostridia begin to thrive. These organisms metabolize sugars and lactic acid to produce butyrate (Figure 1.2) and a mixture of lesser products including acetate, propionate, ethanol, butanol and formate (Thomas and Morrison, 1982). They also degrade amino acids extensively by deamination, decarboxylation and Stickland-type exchange reactions yielding ammonia and a wide range of amines (Table 1.1). Since butyric acid is a weaker acid than lactic acid, and many of the products of amino acid breakdown are bases, clostridial activity slows, or reverses, the normal reduction in pH which occurs in the silo. However, in extreme cases this leads to prolonged fermentation of the crop, excessive nutrient losses and spoilage, and a silage of exceptionally low nutritive value and acceptability to animals (Thomas and Thomas, 1985).

The principal fermentation product of the coliform bacteria is acetate, although they also produce lactate, ethanol, and 2,3-butanediol and may, in part, be responsible for the appearance of these compounds in silage. In addition, coliforms are able to deaminate and decarboxylate amino acids but possess only weak proteolytic properties (Woolford, 1984).

Although yeasts play a lesser role compared with bacteria in grass silages, the active presence of yeasts in silage is considered undesirable on two counts. Firstly, they are associated with aerobic deterioration of silage and secondly they compete with lactic acid bacteria for sugars which they ferment mainly to ethanol (Figure 1.3) which has little, if any, preservative value in silage.

The efficiency of the ensilage process depends on the type of preservation (Table 1.2). Homolactic fermentations are more efficient since they yield products of the maximum acidity with little loss of dry matter or energy. Heterolactic fermentations are less efficient, giving acid and neutral products, and a higher loss of dry matter (DM), although energy loss differs little from that with homolactic fermentation because some of the fermentation products have a higher energy content. Clostridial and yeast fermentations are especially inefficient (Thomas and Morrison, 1982; Figure 1.3).

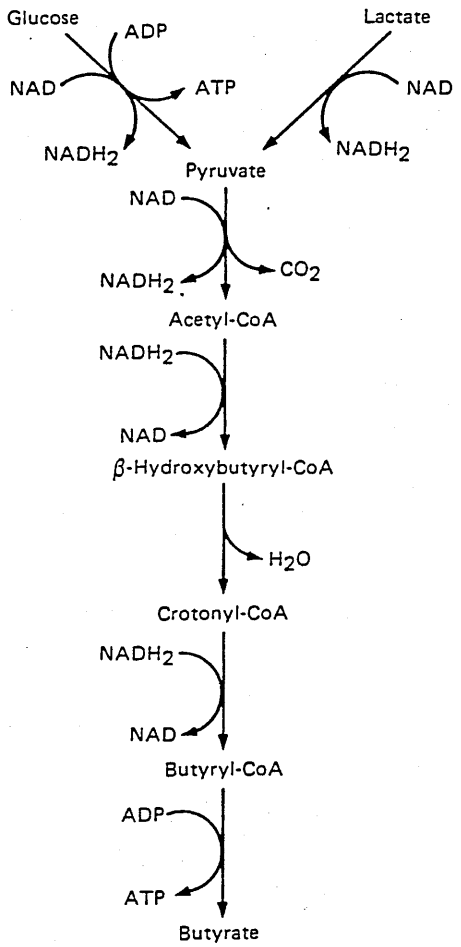


Figure 1.2 Fermentation of glucose and lactate by the saccharolytic clostridial bacteria. (After McDonald, 1981)

Table 1.1 Catabolism of amino acids and amides by proteolytic clostridia (After Mc Donald, 1981)

1. Deamination
   - Arginine → citrulline + NH_3
     - ↳ ornithine + NH_3 + CO_2
   - Aspartic acid → fumaric acid + NH_3
     - ⟶ acetic acid + pyruvic acid
   - Glutamic acid → mesaconic acid + NH_3
     - ↳ acetic acid + pyruvic acid
   - Histidine → urocanic acid + NH_3
     - ⟶ formiminoglutamic acid → formamide + glutamic acid
   - Lysine → acetic acid + butyric acid + $2NH_3$
   - Methionine → α-ketobutyric acid + methylmercaptan + NH_3
   - Phenylalanine → phenyl propionic acid + NH_3
   - Serine → pyruvic acid + NH_3
   - Threonine → α-ketobutyric acid + NH_3
   - Tryptophan → indolepropionic acid + NH_3
   - Tyrosine → *p*-hydroxyphenyl propionic acid + NH_3
   - Asparagine → aspartic acid + NH_3
   - Glutamine → glutamic acid + NH_3

2. Decarboxylation
   - Arginine → ornithine → putrescine + CO_2
   - Aspartic acid → alanine + CO_2
   - Glutamic acid → γ-aminobutyric acid + CO_2
   - Histidine → histamine + CO_2
   - Lysine → cadaverine + CO_2
   - Phenylalanine → β-phenylethylamine + CO_2
   - Serine → ethanolamine + CO_2
   - Tryptophan → tryptamine + CO_2
   - Tyrosine → tyramine + CO_2

3. Oxidation/Reduction (Stickland)*
   (a) Oxidation
   - Alanine + $2H_2O \xrightarrow{-4H}$ acetic acid + NH_3 + CO_2
   - Leucine + $2H_2O \xrightarrow{-4H}$ isovaleric acid + NH_3 + CO_2
   - Isoleucine + $2H_2O \xrightarrow{-4H}$ α-methyl butyric acid + NH_3 + CO_2
   - Valine + $2H_2O \xrightarrow{-4H}$ isobutyric acid + NH_3 + CO_2

   (b) Reduction
   - Glycine $\xrightarrow{+2H}$ acetic acid + NH_3
   - Proline $\xrightarrow{+2H}$ δ-amino valeric acid
   - Ornithine $\xrightarrow{+2H}$ δ-amino-valeric acid + NH_3

* Some examples.

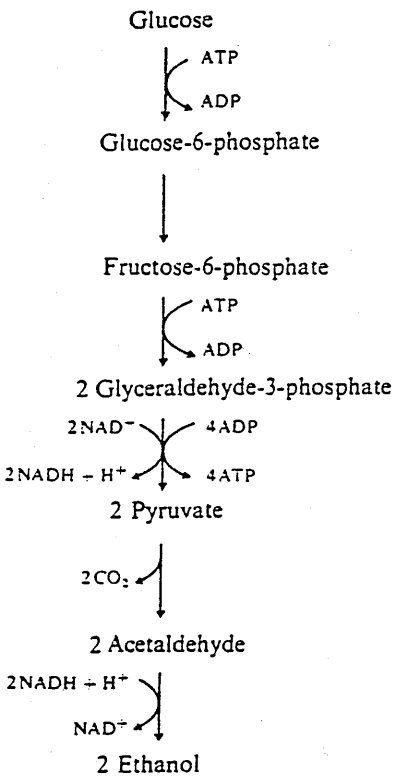


Figure 1.3 Fermentation of glucose by yeasts. (After McDonald, 1981)

Table 1.2 Dry matter and gross energy losses calculated from some important fermentation pathways (After McDonald, 1981)

		Loss (%) DM	Loss (%) Energy
A. Lactic acid bacteria*			
HO	glucose / fructose + 2 ADP → 2 lactate + 2 ATP	0	0.7
HE	glucose + ADP → lactate + ethanol + CO_2 + ATP	24	1.7
HE	3 fructose + 2 ADP → lactate + acetate + 2 mannitol + CO_2 + 2 ATP	4.8	1.0
HO HE	2 citrate + H_2O + ADP → lactate + 3 acetate + 3 CO_2 + ATP	29.7	+1.5
HO HE	malate → lactate + CO_2	32.8	+1.8
B. *Clostridia*			
	2 lactate + ADP → butyrate + 2 CO_2 + 2 H_2 + ATP	51.1	18.4
C. yeasts			
	glucose + 2 ADP → 2 ethanol + 2 CO_2 + 2 ATP	48.9	0.2

* HO = Homofermentative lactic acid bacteria
HE = Heterofermentative lactic acid bacteria

It is clear that rapid attainment of a low pH and anaerobic conditions are crucial to the conservation of nutrients. However, certain modifications in crop composition are inevitably associated with the ensilage process. There is a reduction in WSC content and an accumulation of fermentation end-products, which reflect the nature and extensiveness of the fermentation process. There is also some degradation of hemicellulose (Dewar, McDonald and Whittenbury, 1963; Morrison, 1979) and a marked redistribution of the plant nitrogenous fractions, reflecting hydrolysis of plant protein, and some degradation of amino acids (Brady, 1960).

1.1.2 Manipulation of Silage Fermentation

Although acceptable from the preservation point of view, the product of a natural lactate fermentation may have certain undesirable nutritional characteristics when compared with the original parent material (Table 1.3). Lactate silages are characterized by high concentrations of lactic acid and the nitrogenous components in the silage are mainly in a soluble non-protein form, whereas in fresh forages about 800 to 860 g/kg total N is present as protein. Residual protein N levels are usually between 250 and 450 g/kg Total N and most of the non-protein N (NPN) is present in the form of amino acids (McDonald, 1981). However, when animals consume lactate silage in which the nitrogenous compounds are in highly soluble forms, there are a number of disadvantages in its utilization. The nitrogen in these silages is extremely rapidly broken down in the rumen, resulting in excessive absorption of ammonia from the rumen and consequently microbial protein synthesis may be reduced owing to a lack of synchronization of energy and nitrogen sources (Chamberlain, Martin and Robertson, 1989). It would be appear sensible to consider methods of manipulating fermentation in the silo that not only inhibit undesirable groups of microorganisms, but also produce a silage that is utilized more effectively by the ruminant.

Table 1.3 An example of typical composition of fresh grass and lactate silages made from perennial ryegrasses (After McDonald, 1981)

	Fresh grass	Silage
pH	-	3.92
Dry matter, g/kg	177	190
Organic matter, g/kg DM	930	932
Buffering capacity, mEq/kg DM	350	1120
Total N, g/kg DM	22.7	23.0
Ammonia N, g/kg TN	5	89
Amino N, g/kg TN	39	264
Protein N, g/kg TN	834	333
Water soluble carbohydrate g/kg DM	177	10
Lactic acid, g/kg DM	-	102
Ethanol, g/kg DM	-	12
Glucose, g/kg DM	44	2
Fructose, g/kg DM	29	3
Fructosan, g/kg DM	64	1
Acetic acid, g/kg DM	-	36
Propionic acid, g/kg DM	-	2
Butyric acid, g/kg DM	-	1

1.1.2.1 Wilting

Ensiling crops with high moisture contents encourages a clostridial fermentation because the critical pH value below which clostridial growth is inhibited depends on the moisture content of the plant material, being lower with wetter material. The effect of wilting on silage fermentation is explained by the relatively greater tolerance of lactic acid bacteria to low moisture availability than the Clostridia. Thus wilting of grass to DM contents between 200 and 250 g/kg prior to ensiling has been recognized as an effective means of improving the fermentation quality of silage, particularly where an effective additive has not been used. The effect of decreasing the moisture content on the activity of the lactic acid bacteria and other organisms is reflected in generally higher pH values, frequently accompanied by slightly higher residual WSC levels and lower levels of lactic acid. Wilkins (1984) concluded that wilting reduces the concentration of fermentation acids but increases the proportion of lactic acid in the mixture and reduces ammonia-N concentration. However, there appear to be only little changes in the proportion of N appearing as protein-N (Jackson and Forbes, 1970) and the inhibitory effect on proteolysis which is achieved at high DM levels e.g. with hay, is slight at DM values of 250 to 300 g/kg (McDonald, 1983).

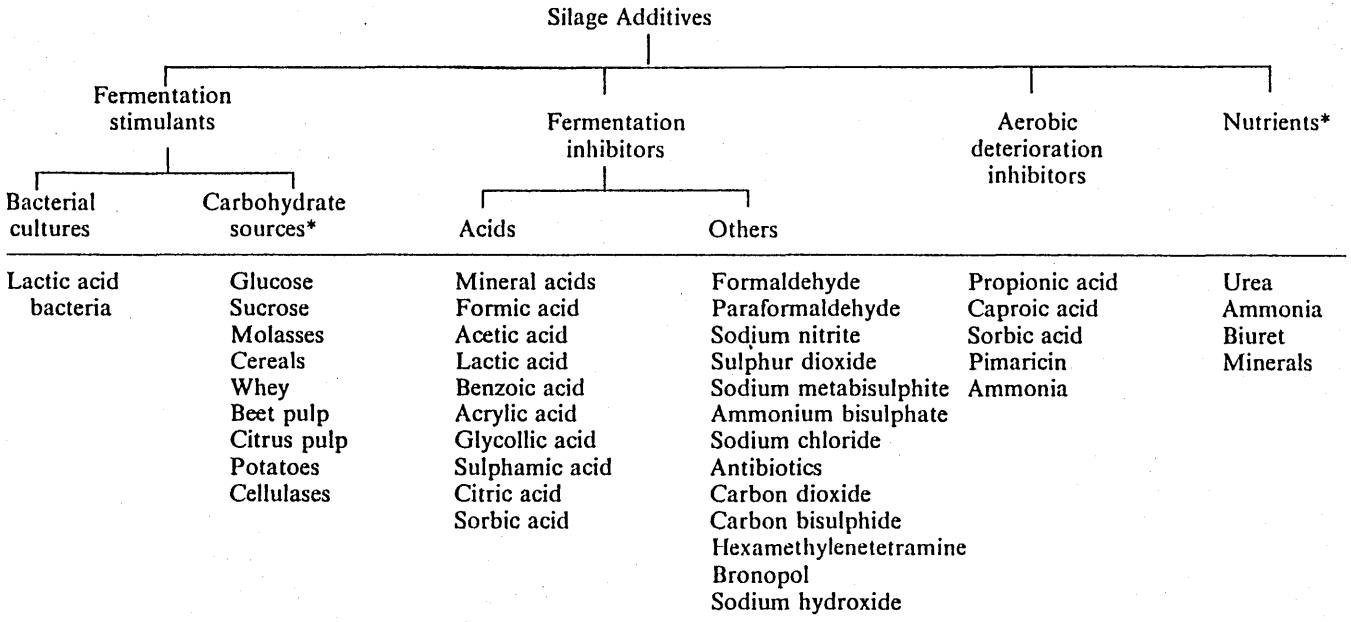
1.1.2.2 Silage Additives

The classification of additives and their mode of action has been reviewed by McDonald (1981) and Woolford (1984). The classification of additives is shown in Table 1.4. It is intended here to discuss briefly the effect of formic acid, sulphuric acid, formaldehyde, mixtures of formaldehyde and acids, and inoculants and enzymes.

1.1.2.2.1 Formic Acid

Unlike inorganic acids, the effect of formic acid on silage fermentation is due in part to its acidic nature and in part to its selective antimicrobial properties, the latter being considered to be linked to the undissociated rather the dissociated molecule. The addition of formic acid to crops at modest levels (1.5-3.0 l/t) rapidly reduces the pH thereby inhibiting the activities of

Table 1.4 Classification of silage additives (After McDonald, 1981)



* Most substances listed under carbohydrate sources can also be listed under nutrients

coliforms and Clostridia. At higher levels (>6.0 l/t) of application the fermentation by lactic acid bacteria is also inhibited and the water soluble carbohydrate contents in the silage may even be greater than those of the original herbage, presumably because of hydrolysis of polysaccharides occurring during the storage period. However, formic acid does not inhibit the growth of yeasts and this may often result in silages with high ethanol contents, a condition which may lead to increased dry matter losses (McDonald, 1983). Extensive reviews of the effects of silage additives on fermentation have been undertaken by Waldo (1978) and Thomas and Thomas (1985). They have concluded that formic acid induces a restriction in fermentation combined with lower concentrations of lactic, acetic and butyric acids, and reduced proteolysis in the resulting silage. Although Chamberlain, Thomas and Wait (1982) reported that application of formic acid at the rates of 0, 2.3, 4.6 and 5.9 l/t reduced the proportion of NPN progressively from 560 to 400 g/kg total N, with commercial application rates in UK (ca. 2.5 l/t) protein breakdown remains extensive (McDonald, 1983).

1.1.2.2.2 Sulphuric Acid

Sulphuric acid has no antimicrobial effect and it was used initially with hydrochloric acid in the AIV process (Virtanen, 1933), where preservation was achieved by direct acidification and large volumes (55-70 l/t) of mineral acid mixtures were applied after dilution with water (1 part acid to 6 parts water) to reduce pH below 4.0. However, the high rate of acid use in this system with resulting detrimental effects on silage intake and concerns over operator safety resulted in this approach not being widely adopted in the UK. However, the recent work of Flynn and his colleagues in the Republic of Ireland, using lower levels of sulphuric acid, has resulted in sulphuric acid being accepted as a cheaper alternative (See Gordon, 1989a). Although Flynn and O'Kiely (1984) observed similar improvements in fermentation characteristics with formic acid (850 g/kg) and with sulphuric acid (450 g/kg) when both were applied at 2.3 l/t, in a series of experiments, Kennedy (1990) observed that sulphuric acid was less effective than formic acid in terms of its ability to enhance the fermentation characteristics and production responses with finishing cattle.

1.1.2.2.3 Formaldehyde and Mixtures of Formaldehyde and Acid

Although the use of acids results in restriction of fermentation and inhibition of clostridial activity, these additives cannot improve the nutritional value of the silage above that of the original forage. In contrast, formaldehyde has an ability firstly to restrict fermentation and secondly to bind with plant proteins (Barry, 1976) and so reduce the rate of breakdown of protein both in the silo and in the rumen, so that the supply of amino acids to the animal can be greater from the silage than from the original herbage (Thomas and Thomas, 1985). However, controlling these processes is extremely difficult. At low levels of application (3 to 5 kg/t), Clostridia appear to be stimulated, whereas high levels may result in an irreversible bonding with protein. This latter aspect reduces protein digestibility and results in decreased protein utilization by the animal. For these reasons, formaldehyde is generally considered to be most effective when used at low levels in a mixture with a normal rate of acid (McDonald, 1983). Valentine and Brown (1973) and Barry (1976) observed a synergistic effect between the components of the mixture and concluded that formaldehyde tended to be more effective in reducing fermentation acids and nitrogen degradation in the presence of formic acid.

1.1.2.2.4 Inoculants and Enzymes

In view of the low numbers of lactic acid bacteria on the standing crop, the provision of efficient acid-producing cultures of these organisms has been considered another way of ensuring an efficient preservation. Criteria for a successful inoculum (Woolford, 1984) include: high growth rate and ability to dominate other organisms; homofermentation; tolerance of acid; ability to ferment fructose, glucose, fructosans and pentosans; inability to produce dextrans; absence of proteolytic enzymes. With laboratory-scale silos, Seale and Henderson (1984) showed that, compared with an untreated control, a commercially prepared *Lactobacillus plantarum* promoted a more rapid drop in the pH of perennial ryegrass and resulted in a silage of lower ammonia-N content. However, in farm-scale operation the results were less clear cut (Gordon, 1989a). The effectiveness of inoculants depends on the availability of soluble carbohydrate substrates in the crop and, in practice, variability in fermentation quality can be

expected to arise from differences in crop composition. In this respect, the development of additives containing hemicellulolytic and cellulolytic enzymes could have potential provided that the hydrolytic activities of these enzymes are maintained in the silo environment (Thomas and Thomas, 1985).

Cell wall degrading enzymes could be used to improve silage fermentation by providing the extra fermentable substrates resulting from the hydrolysis of hemicellulose and cellulose in the crop. Sufficient supply of substrate may result in a rapid acidification hence reducing the risk of a clostridial fermentation and inhibiting the growth of other undesirable micro-organisms and avoiding other detrimental changes (Woolford, 1984). Moreover, another consequence of the addition of these enzymes before ensiling could be a disintegration of cell walls, which might increase the extent and rate of degradation in the rumen and could result in a higher digestibility and nutritive value (McHan, 1986). Recently enzymes, either on their own or in combination with bacterial inoculants, have been examined as a means of releasing suitable energy substrates. Some small scale studies have indicated beneficial effects on rate of silage fermentation when cell wall degrading enzymes have been added either with or without an inoculant (Merry and Braithwaite, 1987; Henderson, McGinn and Kerr, 1987). In a production trial with dairy cows, Chamberlain and Robertson (1989) treated silage with various mixtures of cellulase and hemicellulase and observed reduced NDF (neutral detergent fibre) and ADF (acid detergent fibre) contents in the silage. Further, enzyme treatment significantly increased the digestibility and intake of silage, and increased milk production.

1.1.3 Characteristics of Silage as a Feed

The main effects of ensilage on herbage composition are the loss of herbage WSC and the associated formation of fermentation end-products and the breakdown of plant protein. With respect to breakdown of plant protein, typically in well-preserved lactate silages made without additives, or in silages made with acidic silage-additives applied at conventional UK rates, NPN accounts for 400-650 g/kg total N but NH_3-N is only 50-100 g/kg total N. In silages prepared with formaldehyde additives, or with acidic additives used at high application rates, NPN may

be as low as 200-400 g/kg total N with NH_3-N being 30-80 g/kg total N. With poorly preserved silages, NPN is generally 550-750 g/kg total N and NH_3-N may be as high as 300 g/kg total N, depending on the degree of amino acid breakdown (Thomas and Chamberlain, 1982a).

1.2 Consequences of Changes in Chemical Composition for Nutritional Value

A small number of experiments have been conducted to evaluate the potential of silage alone for milk production. With mid-lactation Ayrshire cows given formic acid treated silage as a sole feed, Castle (1982) reported average intakes of 11.3kg DM/day and mean milk yield of 14.4 kg/day but the cows lost body weight substantially. He concluded that DM intake is the major limitation to milk production from silage. More recently, Rae *et al.* (1987) have conducted a series of five trials to investigate the effect of offering formaldehyde-sulphuric acid treated silage *ad libitum* to Friesian cows and heifers in early lactation for 87 days. DM intakes averaged 12.6kg DM/day, mean milk yield of cows was 21.1 kg/day and corresponding total liveweight loss was 46kg. It must be noted that these levels of production were achieved at the expense of considerable loss of body weight. Cows given diets of silage alone produce milk at a level well below their potential owing to limitations on silage intake compounded by a reduced efficiency of utilization of silage nutrients.

1.2.1 Intake

In all species of animals, voluntary food intake is regulated centrally through the activity of the hypothalamus where information on the animal's requirements for nutrients, in particular for energy, is integrated with information on what the animal derives about its food. This integration is achieved through the action of central nervous system receptors that respond to the sensory qualities of foods (taste, smell, texture), to the physical effects of food ingestion on the gut (stretch, pressure), to chemical stimuli arising from the end-products of digestion before and after their absorption, and to any intake-depressing compounds present in the food (Figure 1.4).

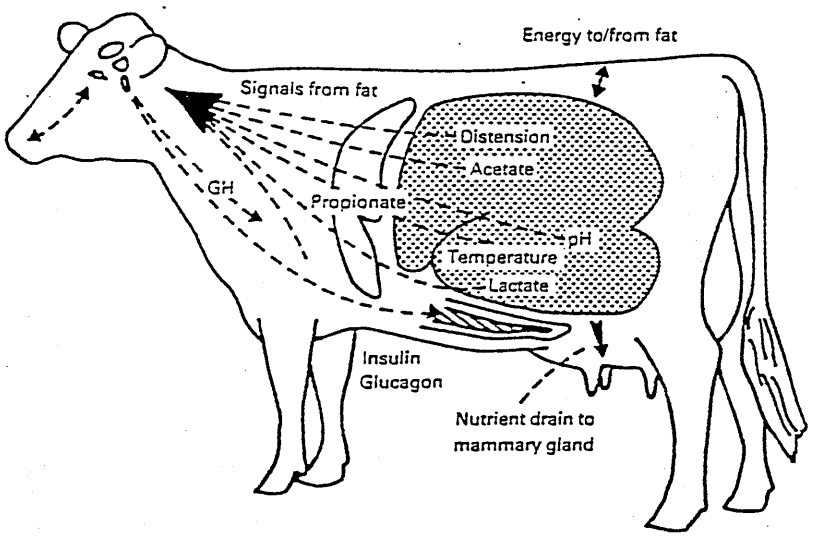


Figure 1.4 Schematic representation of the control of voluntary food intake in the dairy cow. (After Forbes, 1986)

For ruminant animals consuming low-energy forages (e.g. hays and straws) intake is regulated predominantly by physical factors, principally 'physical fill' in the rumen, whilst for high energy forages and concentrates intake is regulated by physiological factors, principally through the animal's perception of the volatile fatty acid (VFA) produced by ruminal fermentation. In a summary of experiments with lactating cows given rations with DM digestibilities between 52% (all forage) and 80% (all concentrate), Conrad, Pratt and Hibbs (1964) suggested that physical factors were important in forages having a DM digestibility of less than 67%, but that with higher quality feeds, physiological factors become important. For experimental diets consisting of cereal concentrates with various proportions of indigestible diluents, Baumgardt (1970) concluded that the point of change from physical to metabolic regulation of intake occurred at a dietary ME concentration of approximately 9 MJ/kg DM (Figure 1.5).

However, Campling (1970) quoted instances, e.g. silage, where intake of feeds with DM digestibility less than 66% did not appear to be primarily limited by the amount of material in the reticulo-rumen. Campling (1966) and Waldo *et al.* (1966) reported that DM content of the rumen was higher with hay than with silage feeding when animals were offered hay or silage of similar digestibilities. The DM digestibilities of the forages used in these experiments ranged from 53 to 72% and lower values should have been within the range where intake is limited by physical factors (Conrad *et al.*, 1964). These results suggest, therefore, that factors other than physical fill may be particularly important in limiting silage intake.

Although factors other than physical fill may be particularly important in controlling silage intake, there is evidence that even for highly-digestible silage, physical fill is also important (Thomas and Chamberlain, 1982b). Farhan and Thomas (1978) observed a 16.5% reduction of intake when water-filled bags were inserted into the rumen of the cows fed well-preserved, highly-digestible silage. Several workers have shown conclusively that silage intake can be increased by mincing the silage before feeding (Thomas, Kelly and Wait, 1976; Deswysen, Vanbelle and Focant, 1978) and reducing the chop-length during silage-making (Castle, Retter and Watson, 1979; Castle, Gill and Watson, 1981), although it is important to

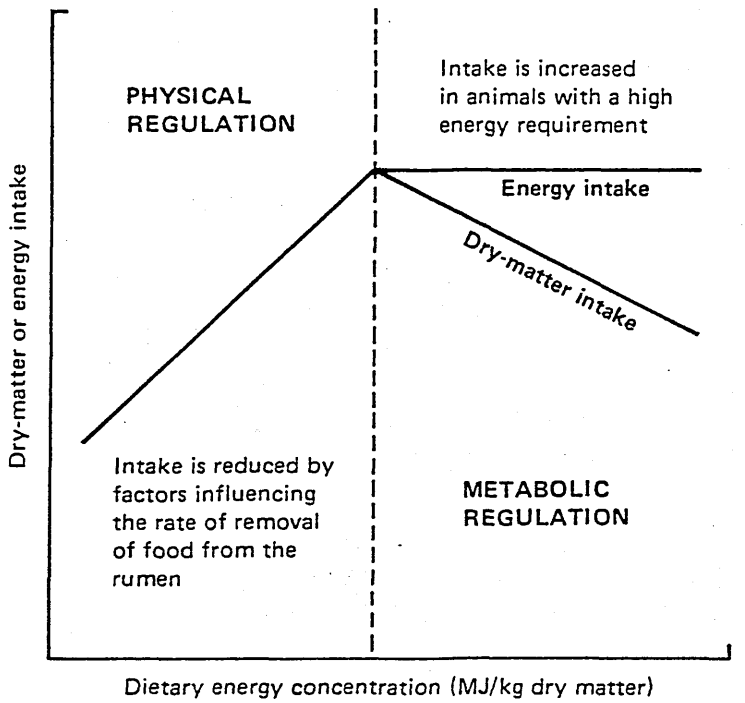


Figure 1.5 A schematic representation of the relationship between voluntary food intake in the cow and the concentration of energy in the diet. (After Thomas and Chamberlain, 1982b)

separate the influence of chop-length *per se* from the indirect effect through silage fermentation quality (Thomas and Thomas, 1985).

Silage is generally consumed to a lesser extent than the corresponding fresh forage or hay of similar digestibility (Moore, Thomas and Sykes, 1960; Brown *et al.*, 1963). For example, with lactating cows given silage and hay prepared from the same sward, Murdoch and Rook (1963) recorded intakes of 14.4kg DM/d with hay and 9.06kg DM/d with silage. However, the extent of the depression varies greatly. Demarquilly (1973) noted a range from -1 to -64% compared with fresh grass and this undoubtedly reflects the range in the changes in the chemical composition during ensilage.

The eating and ruminating behaviour of animals given silages is also different from that of animals given fresh or dried forage. When given silages, animals spend less time per day eating and eat a greater number of small meals. The incidence of pseudo-rumination is also increased (Dulphy and Dermarquilly, 1973; Deswysen *et al.*, 1978; Castle *et al.*, 1979). The cause of the behavioural pattern observed with silage diets has not yet been fully elucidated but Clancy, Wangsness and Baumgardt (1977) reported that intraruminal infusion of lucerne silage juice reduced DM intake, rumen motility and the rate of eating in sheep given hay. This has been confirmed in studies with grass silages treated with formic acid (Smith and Clapperton, 1980). This indicates that the feeding behaviour of animals given silage diets is influenced by the fermentation end-products or unidentified compounds, possibly amine(s), present in silage juice (Clancy *et al.*, 1977; Thomas, Kelly and Chamberlain, 1980). However, it is interesting to note that Steen (1986) reported that supplementation with silage effluents, which were collected from silages treated with formic or sulphuric acids at levels ranging from 2.0 to 3.0 l/t, did not reduce silage intake. Attempts have been made to clarify the causes of the depression in intake, either by a statistical approach or by direct experimental investigations.

1.2.1.1 Statistical Approach

It has been considered that non-physical factors appear to be particularly important in controlling silage intake. Since some of the end-products of silage fermentation are also the

end-products of microbial fermentation in the rumen and those are known to influence intake, a study to correlate the low intake of silage and its components has been conducted (Gill, Rook and Thiago, 1988). In the relationship between digestibility and intake, Castle (1982) and Gordon (1989b) reported a mean increase in silage DM intake corresponding to 1% unit increase in D-value of 0.24 and 0.16kg respectively. In an analysis of experiments that involved the feeding of well-fermented silage of different digestibilities to dairy cows, Thomas (1980) also obtained a positive relationship between digestibility and intake. However, marked differences were also observed in intake between experiments, and differences between silages in fermentation patterns can have an important effect in modifying the response in intake to increased digestibility (Thomas and Thomas, 1985).

A number of workers have used regression analysis to correlate the concentration of specific silage components with voluntary intake in sheep and cattle (Wilkins *et al.*, 1971; Wilkins *et al.*, 1978). However, there have been fewer studies on the effect of silage fermentation end-products on voluntary intake of lactating dairy cows. Lewis (1981), using a regression approach, found that only ammonia-N as a proportion of the total N had a significant negative effect on intake, although both DM content and DM digestibility had positive effects. Using a multiple regression approach, Gill *et al.* (1988) analysed data from a number of trials conducted at the Institute for Grassland and Animal Production. They concluded that further analysis of dairy cow intakes confirmed earlier work with sheep and lower levels of ammonia-N (g/kg total N) had a negative effect on intake while acetic acid tended to have a linear negative effect. Lactic acid *per se* had a negative effect, but when expressed relative to total acids the effect was positive. Statistical studies of this type provide useful information but they can suffer the shortcoming of collinearity among silage constituents. To overcome this, Rook, Gill and Dhanoa (1989) have used ridge regression techniques. Although some improvement was observed over existing models, errors of prediction were still high. While low intake may be associated with a particular type of silage fermentation, it is not possible in this type of study to ascribe a causative intake-depressing action to specific silage components. Thus direct investigations of the effects of individual

fermentation products have been undertaken.

1.2.1.2 Ruminal Infusion and Dietary Addition of Silage Fermentation Products

1.2.1.2.1 Organic Acids

On silage only diets, VFA comprise a major part of the absorbed energy fractions since the quantity of protein absorbed from the small intestine is generally low. A number of workers have found significant decreases in short-term intake response to intraruminal infusion of acetate in sheep (Ulyatt, 1965) and in dairy heifers (Simkins, Suttie and Baumgardt, 1965; Bhattacharya and Warner, 1968) when animals were given silage. Dietary supplements of acetic acid also have generally reduced silage intake significantly (Hutchison and Wilkins, 1971). It has been considered that acetate may limit intake either directly through an effect on receptor sites within the rumen (Baile and Mayer, 1970; Forbes, 1980) or indirectly via a reduced rate of clearance from the tissues. Since the successful disposal of acetate requires an adequate and balanced supply of glucose (MacRae and Lobley, 1982), with silage diets, where potential supply of glucose is considered to be generally low, problems of acetate disposal within the tissues may occur. In an experiment to examine the interaction between acetate and glucose on intake, Gill, Sargeant and Beever (unpublished observations; cited by Gill *et al.*, 1988) showed a strong negative intake response to increasing levels of acetate infusion into the rumen, but when 15 g/kg LW of glucose was infused into the duodenum, a substantial alleviation of this effect was observed. Intraruminal infusion of propionate has been shown to depress intake (Simkins *et al.*, 1965). However, Bhattacharya and Warner (1968) reported that addition of citrate with the acetate partially alleviated the depression of intake by acetate, while replacing part of the acetate by an equicaloric amount of propionate prevented the depression. They concluded that the beneficial effect of propionate could be due to its glucogenic properties.

The level of lactic acid in the silage is often 10-15% of the dry matter and while this may be beneficial with respect to silage preservation, the consequences for rumen metabolism, and hence silage intake, may be greater than previously considered (Beever and Gill, 1987).

Chamberlain, Thomas and Anderson (1983) and Gill *et al.* (1986) demonstrated that in silage-fed animals both L(+) and D(-) isomers were rapidly (with a half life of about 25 minutes) fermented in the rumen to acetate, propionate and butyrate, the proportions of the acids formed varying with the relative numbers of bacteria and protozoa in the rumen. Although it is difficult to establish direct links between high concentrations of lactic acid and reduced voluntary intake of silage, there is some quantitative information on the capacity of the rumen to metabolize lactic acid. From the experiments using intraruminal infusions of lactic acid, Newbold, Chamberlain and Williams (1986) suggested that if the lactic acid concentration in silage reached around 200 g/kg DM, the capacity of rumen could be exceeded, leading to indigestion and reduced feed intake. Thomas, Gill and Austin (1980) observed a depressive effect on silage intake by lactic acid addition when grass silage was offered either with or without the addition of lactic acid (50 g/kg silage DM). More directly, using intraruminal infusion of lactic acid, Cole and Gill (1990) concluded that the effect of lactic acid infusion appeared to be a short-term response. Moreover, Thomas *et al.* (1980) also noted that the depressant effect of lactic acid on silage intake was overcome by a dietary supplement of fishmeal.

1.2.1.2.2 Nitrogenous Compounds

As a consequence of protein degradation in the silo, and extensive protein breakdown in the rumen, substantial quantities of ammonia may be absorbed from the rumen. This could be important in the control of intake of grass silages with high N contents and high proportions of NPN. Intraruminal infusions of ammonium salts have reduced silage intake (Thomas *et al.*, 1961) and resulted in shorter and less frequent meals in animals given high-concentrate diets (Conrad, Baile and Mayer, 1977). These intake effects appear to relate to an excessive uptake of ammonia from the rumen, and recently, Siddons *et al.* (1985) showed that absorbed ammonia-N could be almost 50% of total N intake on a grass silage diet. Apart from the consequence of this for the supply of protein to the small intestine, an elevated portal supply of ammonia may have a significant effect on metabolism in the liver and the peripheral tissues

(Symonds, Mather and Collis, 1981; Beever and Siddons, 1986). However, the quantitative significance of this on voluntary feed consumption is as yet unclear.

The concentration of ammonia-N in the silage has usually been used as an index of poor silage fermentation. Clancy *et al.* (1977) attributed 40% of the depression in intake when silage extract was infused into the rumen to ammonia, and Barry, Cook and Wilkins (1978) reported that ammonia as a proportion of the total N accounts for 37% of the variation in organic matter intake in sheep offered silages. In extensively fermented silages, significant quantities of amines may be formed during ensilage. Barry *et al.* (1978) concluded that the production of potentially toxic amines through decarboxylation rather than deamination reactions was more likely to be the prime factor limiting intake. Studies on the effects of nitrogenous silage fermentation products have indicated that intake is not reduced by dietary or intraruminal administration of tyramine, tryptamine (Neumark, Bondi and Vocani, 1964), histamine (McDonald, Macpherson and Watt, 1963) or γ-amino butyric acid (Buchanan-Smith, 1982). Recently, however, Buchanan-Smith and Phillip (1986) reported that intraruminal infusion of mixtures of nitrogenous silage constituents or γ-amino butyric acid depressed cumulative intake up to 8 h following feeding. Although these attempts to relate intake to the end-products of fermentation have produced variable and equivocal results with no clear picture emerging, the nitrogenous constituents produced during ensilage appear to contribute to the short-term control of intake.

The data reviewed suggest that the inhibition of intake could not be attributed to one constituent, but was due to the combined effect of several, including both the organic acids and the nitrogenous constituents. The main mechanism of response to specific silage constituents is thought likely to be mediated through chemo- or osmoreceptors in the rumen, duodenum or liver (Gill *et al.*, 1988).

1.2.2 Digestion and Utilization of Nutrients

1.2.2.1 Digestion and Utilization of Silage Energy

Fermentation normally leads to an increase in gross energy (GE) content of around 1.6 MJ/kg DM (McDonald and Edwards, 1976). The increases in GE are due mainly to losses of DM without concomitant losses of energy (McDonald, 1981). Thomas and Chamberlain (1982b) reported a value of 18.6 with a range of 17.5-20.0 MJ/kg DM for formic acid treated silages and Wainman, Dewey and Boyne (1979) observed 18.4-19.5 MJ/kg DM for grass and grass-clover silages.

Of the large proportion of silage digestible energy (DE) digested in the rumen, most is absorbed as VFA and rumen VFA production with silage diets accounts for about 0.53 of the DE (Thomas *et al.*, 1980c). In animals fed twice daily, the composition of the mixture of VFA present shows a pronounced change after feeding with a marked peak in the proportion of propionic acid due to fermentation of lactic acid in the rumen. Further, this fermentation activity is located principally in the protozoal rather than bacterial fraction of rumen digesta and when animals were defaunated the post-feeding peak in propionate was reduced and there was an increase in the proportion of butyrate in the fermentation products (Chamberlain *et al.*, 1983). Although it would be appear that an efficient conversion of sugar to lactic acid in the silo represents no apparent energy loss to the ruminant since it is provided with acetate and propionate for its own metabolism, the rumen microorganisms are faced with a substantial loss in ATP supply.

There is evidence that the ensiling process influences the efficiency of utilization of ME by non-lactating ruminants. In growing cattle given a range of silages of good fermentation quality, determined efficiency of utilization of ME for maintenance (k_m) was lower than predicted using the equation of Agricultural Research Council (ARC, 1980); on average the difference was approximately 5% of predicted value. However, determined efficiency of utilization of ME for fattening (k_f) showed more marked differences than for k_m, the difference being 17.3% of the calculated value (McDonald, 1983). The low efficiencies and the variability between silages have been suggested to arise from an imbalance between energy and

protein supply, which may be related to the extent of silage fermentation. Alternatively, the effects on thermogenesis could be associated with the presence of specific flavonoid components in silage (Thomas and Thomas, 1985).

In contrast, relatively little information is available on the efficiency of utilization of silage energy for milk production. Very few calorimetric determinations have been made with cows receiving grass silage diets. Unsworth, Wylie and Anderson (1984) and Unsworth and Gordon (1985) have conducted calorimetric evaluations with wilted and unwilted silages given with approximately 40% concentrate and observed k_{10} (efficiency of utilization of ME for lactation at zero body weight change) values of 0.58 and 0.56 respectively. These compared with values of 0.64 and 0.65 estimated from ARC (1980). Analysis of feeding experiments where silages were given as the sole feed (Rae *et al.*, 1987) indicates the efficiency of utilization of ME for lactation (k_1) values ranging from 0.54 to 0.58 compared with predictions of 0.61 to 0.63. Thomas and Castle (1978), on the basis of their feeding trials, have calculated k_1 values of between 0.39 and 0.64 for diets containing a high proportion of silage. Although such estimates are subject to considerable error, it has been suggested that the low k_1 values for silage diets are related to an imbalance in energy and protein supply (Thomas and Rae, 1988) and it is noteworthy that Thomas and Castle (1978) and Chamberlain *et al.* (1989) found k_1 values to be consistently higher with silage diets containing supplementary protein.

1.2.2.2 Digestion and Utilization of Silage Nitrogen

1.2.2.2.1 Degradation of Silage N in the Rumen and Absorption of Ammonia from the Rumen

The rate of degradation of silage nitrogen in the rumen is characteristically high and varies with the additive used during ensilage. When formaldehyde is applied at a high rate, degradability is reduced. Based on a survey of data from the literature, Thomas (1982) estimated that the rumen-degradability of silage N *in vivo* was 0.78-0.86 for silages made without additives, 0.54-0.81 for formic-acid silage and 0.31-0.51 for silages made with the addition of formaldehyde or formic acid/formaldehyde (35-60g formaldehyde/kg protein).

However, these values should be interpreted with caution. Although results with formic acid silages (Thomas *et al.*, 1980c) indicated that the amount of undegraded silage protein passing to the duodenum was closely related to the proportion of true protein in the silage crude protein, Chamberlain *et al.* (1982) observed that formic acid treatment, which increased the proportion of true protein in the silage by limiting protein breakdown in the silo, merely resulted in an increased proportion of silage true protein being degraded in the rumen with no net benefit in the passage of protein to the small intestine. With respect to the techniques used to measure degradability, it would appear that the *in sacco* tests may overestimate the degradability of formaldehyde treated silages and that some of the degradability values determined *in vivo* for formic acid silages may be underestimates (Thomas and Thomas, 1985).

Silage nitrogen is rapidly broken down in the rumen, resulting in high concentrations of ruminal ammonia. McDonald and Edwards (1976) reported that peak ruminal ammonia concentrations for six lactate silages given to sheep varied from 195 to 450 mg/l and there was a highly significant correlation between the concentration of ruminal ammonia, and the NPN and ammonia-N contents of the silages. Durand, Zelter and Tisserrand (1968) also obtained peak rumen ammonia-N values ranging from 300-500 mg/l in sheep on silage diets. Rumen ammonia concentration varies both with the nitrogen content of the silage and with the method of silage making. Formaldehyde (Beever *et al.*, 1977) or mineral acids (Durand *et al.*, 1968) tend to reduce rumen ammonia levels, although a consistent relationship between silage NPN and rumen ammonia concentration is not always observed. In the work of Chamberlain *et al.* (1982), referred to earlier, although application of increasing levels of formic acid reduced the proportion of NPN progressively from 560 to 400 g/kg total N, when the silages were given to sheep there were no significant differences in rumen ammonia concentration between treatments.

There is clear evidence of a pronounced net loss of N between mouth and duodenum due to ammonia absorption in the rumen. Thomas and Chamberlain (1982a) analysed published data and found that there was a linear relationship between N intake and N flow to the duodenum but when data were selected to avoid the confounding effects of protein protection by

formaldehyde, there was an indication that net losses of N between the diet and the duodenum would occur consistently as the CP content of the diet was raised above 160 g/kg DM. Thomas and Thomas (1985) also concluded that with diets of moderate or low protein content (<140 g/kg DM), sheep show little net loss of N in the rumen and duodenal non-ammonia nitrogen (NAN) flow approximately equals dietary N intake. However, above this level, net losses of nitrogen start to increase and at high dietary protein levels, net nitrogen loss can account for one-third of the total N ingested. This implies that with silages of lower CP content ammonia losses from the rumen are counterbalanced by the entry of N in endogenous secretions. Taking the endogenous N secretion into account, there must be substantial absorption of silage N from the rumen even with low protein silages (see Thomas and Chamberlain, 1982a). However, with cows net losses of N in the rumen become significant at lower dietary protein concentrations (Chamberlain, Thomas and Quig, 1986). The reason for this difference is unknown, but it is noteworthy that the rumen ammonia concentration in cows appears to be higher than in sheep for a given silage of similar protein content. In nutritional terms the difference is of considerable importance since the indication is that even with silages of moderate protein content, dairy cows may lose up to one-quarter of the nitrogen ingested through absorption of ammonia in the rumen (Thomas and Thomas, 1985). Further, Siddons *et al.* (1985) estimated that absorbed ammonia-N could be almost 50% of total N intake on a grass silage diet. This excessive absorption of ammonia could lead to some perturbations of metabolism. As Armstrong (1981) has suggested, high ruminal ammonia levels may adversely influence the efficiency of energy utilization in two ways. First, the need for an additional energy requirement for urea synthesis and, secondly, detoxification of ammonia in the liver may reduce glucose formation from propionate. A reduction in gluconeogenesis might result in a deficiency of NADP essential for fat synthesis. Symonds *et al.* (1981) reported that the liver of dairy cows could extract up to 15mmol ammonia per min from the portal vein and above this level arterial ammonia concentrations were seen to rise, with quite dramatic clinical changes occurring in the cow. Thus, an elevated portal supply of ammonia may have a significant effect on liver and peripheral tissue metabolism (Beever and Siddons, 1986).

1.2.2.2.2 Microbial Protein Synthesis in the Rumen

There is clear evidence that diets containing a high proportion of silage support a low rate of microbial protein synthesis in the rumen. The Agricultural Research Council (ARC, 1984) adopted a value of 23g microbial N/kg organic matter digested in the rumen (DOMR) for silages as compared with a value of 32g N/kg DOMR for hays and grasses. However, the estimates of microbial efficiency have been widely variable, and this may reflect differences in methodology between laboratories but may also be a function of the characteristics of the silage used in the experiments. High levels of formaldehyde (6 g/100g CP, Beever *et al.*, 1977) but not moderate rates (3.5 g/100g CP, Siddons, Evans and Beever, 1979) have markedly depressed microbial protein synthesis. However, apart from the effects of formaldehyde, the role of fermentation characteristics remains unclear. The extent of proteolysis occurring in the silo seems to be of little consequence since the true protein saved during silage fermentation may represent that portion which in any event is rapidly degraded in the rumen (Chamberlain *et al.*, 1982). Thomas and Rae (1988) concluded that little is known about the consequences of decarboxylation and deamination, but it is likely that their effects are more profound. The cause of the low rates of microbial synthesis with silage diets could be explained partly by low yields of ATP in the rumen from the digestion of silage fermentation products (Thomas, 1982) and the extent of reduction in ATP production depending on fermentation characteristics in the silo. A significant part of the energy extractable from rumen fermentation is extracted already during the ensiling process: up to 150 g/kg DM of the DM in unwilted silage may be present as acetic and/or lactic acid (McDonald, 1981). This may amount to up to 25 to 30% of the organic matter (OM) which would normally be digested in the rumen. Acetate and butyrate will make no contribution to ATP production and lactic acid, the main fermentation product, is fermented in the rumen to propionate and butyrate (Chamberlain *et al.*, 1983) and thus could furnish a net gain in ATP supply. Chamberlain (1987) calculated that the reduction in ATP yield in the rumen with a well-preserved naturally fermented silage (containing lactic and acetic acid of 150 and 40 g/kg DM) was 15-20% less than with silage of restricted fermentation with formic acid (containing lactic and acetic acid of 60 and 20 g/kg DM). It has been estimated

that feeding silage could deprive the micro-organisms in the rumen as much as one third of their energy supply (Tamminga, 1982). However, the rates of microbial synthesis with some silages (Beever *et al.*, 1977; Thomas *et al.*, 1980) are much lower than can be explained simply in terms of the effects of the silage fermentation products, and also there is no clear evidence of a systematic variation in synthesis rate between silages made from the same grass but prepared with differing contents of lactic and acetic acids (Chamberlain *et al.*, 1982).

1.2.2.2.3 Supply of Protein and Amino Acids to the Small Intestine

Because of the low rate of protein synthesis in the rumen, the proportion of microbial protein in the total protein passing to the duodenum in animals given silage diets is lower than is found typically in animals consuming diets based on hay (Thomas and Chamberlain, 1982a). Increasing the level of application of formic acid up to 5.9 l/t did not increase protein flow to the duodenum (Chamberlain *et al.*, 1982). Similarly, additives containing formaldehyde at low rates of application reduced silage NPN, soluble-N and ammonia-N levels but there was no associated increase in duodenal protein flow (Rooke, Brookes and Armstrong, 1983). Hence the rate of application of formaldehyde must be sufficient not only to restrict decarboxylation and deamination of amino acids in the silo but also to protect silage protein from microbial attack in the rumen (Thomas and Thomas,1985). In a comparison with sheep given silages, untreated and pretreated with a mixture of equal volumes of formic acid and formalin applied at a rate of 9 l/t, Siddons *et al.* (1979) found that although the amount of microbial amino acids entering the small intestine was similar for both silages, the quantity of food amino acids reaching the small intestine was significantly higher for the treated silage compared with the control.

There are also some characteristic differences in the amino acid composition of the duodenal digesta between silage- and hay-based diets (Table 1.5). With silage diets, the digesta contain lower concentrations of arginine, lysine and especially methionine (Thomas *et al.*, 1980d; Chamberlain *et al.*, 1986). The coefficient of digestibility of total amino acids in the small intestine of animals receiving silage diets appears to be similar to that observed in

Table 1.5 The proportions of essential amino acids (g amino acid per kg determined amino acids) in the duodenal digesta of animals given diets containing silage or hay (After Thomas and Chamberlain, 1982b)

	Silage diet[+]		Hay diet[++]	
	Mean	s.e.	Mean	s.e.
Histidine	13	3	nd	
Threonine	66	5	60	1
Arginine	38	4	58	2
Methionine	17	1	32	1
Valine	66	1	68	3
Phenylalanine	51	5	nd	
Isoleucine	50	6	59	1
Leucine	80	11	79	2
Lysine	68	4	85	1

[+], mean with s.e. for three diets of ryegrass silage alone, and one diet of ryegrass and barley (800:200 g/kg)

[++], mean with s.e. for three diets consisting of hay and a 500:500 g/kg barley-maize mixture in proportions 1000:0, 700:300 and 400:600 g/kg

nd, not determined

animals given diets of hay and is normally in the range 0.65-0.75 (Harrison *et al.*, 1973; Beever *et al.*, 1977; Thomas *et al.*, 1980d). Silage made with formaldehyde additives may give values at the lower end of these ranges, indicating that formaldehyde can impair the absorption of amino acids in the small intestine (Beever *et al.*, 1977). However, the effect does not appear to become severe until application rates are above 50g formaldehyde/kg grass CP (Thomson *et al.*, 1981). It appears that in terms of the absorption of individual amino acids, lysine is the most affected amino acid and that, even at application rates of 50g formaldehyde/kg CP, absorption of lysine is depressed (Thomas and Thomas, 1985).

1.2.2.2.4 Improving the Utilization of Silage Nitrogen in the Rumen

The relative deficiency in energy supply and the rapidity of release of ammonia from silage N compounds in the rumen argue for supplements of readily-fermentable carbohydrate sources to promote the microbial fixation of ammonia. The efficiency of capture of silage nitrogen in the rumen varies with the amount and type of carbohydrate supplement used and notably is greater with sucrose than with starch (Syrjala, 1972; Chamberlain *et al.*, 1985). Although this difference might be related to the relative rates of fermentation of sucrose and starch in the rumen, an alternative explanation is that the substrates exert their influence via the numbers of rumen protozoa: starch markedly increased protozoa numbers and probably stimulated wasteful intraruminal recycling of nitrogen, so limiting its effect on the efficiency of microbial synthesis (Chamberlain *et al.*, 1983). Thus when barley is chosen as an energy supplement, losses of N across the rumen are not prevented unless barley accounts for around 50% of the total diet DM even with modest concentrations of CP in the silage (Chamberlain *et al.*, 1989). In a series of experiments, Chamberlain *et al.* (1985) observed that supplements of maize starch resulted in a marked increase in protozoal numbers but sucrose, glucose and xylose supplements had no such effect. Xylose was particularly effective at reducing rumen ammonia concentrations, an observation which supports the results *in vitro* of Henderickx and Martin (1963) which suggested that pentoses were superior to hexoses with regard to their ability to promote microbial protein synthesis. The effectiveness of sugar as a supplement to

silage has been further demonstrated by the study of Rooke *et al.* (1987) in which silage was supplemented with glucose syrup, urea, casein or casein plus glucose syrup (Table 1.6). Glucose supplements lowered rumen ammonia-N concentrations, increased flow of NAN to the duodenum and increased the efficiency of microbial protein synthesis.

However, sugar supplements have disadvantages: their rapid rate of fermentation can induce a low rumen pH which, in turn, can restrict microbial growth (Russell, Sharp and Baldwin, 1979). Sodium bicarbonate in combination with sucrose prevented the low rumen pH induced by sucrose alone and markedly improved the effectiveness of the sugar supplement in reducing ammonia concentration (Chamberlain *et al.*, 1985). Subsequently, beneficial responses of duodenal N flow to dietary additions of bicarbonate were observed (Newbold, Thomas and Chamberlain, 1988). Although the mode of action of the bicarbonate on microbial protein synthesis is not clear, Chamberlain *et al.* (1989) suggested that both increased rumen pH and liquid outflow rate have the potential to increase microbial protein synthesis. In a production trial with dairy cows, Rae, Thomas and Reeve (unpublished observations; cited by Thomas and Rae, 1988) reported that molasses plus bicarbonate gave disappointing results but the quantity of sugar supplied by the supplement may have been insufficient to stimulate adequately the microbial incorporation of silage N (Thomas and Rae, 1988).

1.3 Protein and Amino Acid Nutrition in Cows Receiving Silage-Based Diets

In attempting to maximize the utilization of silage for milk production and overcome its nutritional limitations, protein supplementation has received particular attention. In practice, protein supplements increase silage intake and milk production but the precise mechanism is still not clear. It has been suggested that the milk production responses could derive from effects on voluntary intake, ration digestibility and improved amino acid supply (Oldham, 1984).

Table 1.6 The effect of intraruminal infusions of casein, urea and glucose syrup on ruminal digestion in cows given a diet of grass silage (After Rooke, Lee and Armstrong, 1987)

	Infusate				
	Water	**Casein**	**Urea**	**Glucose**	**Casein + glucose**
CP in diet[+], g/kg DM	115	137	139	98	114
Duodenal NAN/N intake, g/g	0.95	0.81	0.76	1.23	1.35
Microbial N at the duodenum, g/d	63	75	68	81	109

[+], including infused nutrients

1.3.1 Effects of Protein Supplementation on Silage Intake

In contrast to carbohydrate and lipid supplements, feeding of groundnut or soya (Castle, 1982) or a mixture of fishmeal and soya (Rae *et al.*, 1986) as the sole supplements, do not reduce silage intake and may even increase it. Furthermore, the inclusion of protein in cereal concentrates reduces substitution rate by an amount that is greater than would be expected simply from the reduction in starch content (Castle, 1982), suggesting that supplementary protein has a stimulatory effect on silage intake. However, the intake responses to increasing the CP content in the diet are extremely variable. Oldham and Alderman (1982) reported a mean increase of 0.34kg with a variable range (-0.24 to 1.35) in response to each % unit increase of CP in the diet for dairy cows as a whole. Gordon, Peoples and Mayne (1982) summarized four experiments and reported an increase of 0.032kg silage DM (range of 0.021 to 0.041) per % unit increase in CP content in the supplement. Although Thomas (1987) suggested that differences in digestibility and fermentation quality could be the cause of the variability, the variability in the response remained even when Chamberlain *et al.* (1989) restricted the data to diets of high digestibility, well-preserved grass silage. They obtained a mean response of 0.29kg with range of 0.12 to 0.51kg.

There is some evidence that the response in intake is modulated by the dietary protein sources, being greater with fishmeal than with vegetable protein sources like soya bean meal (Thomas and Thomas, 1985). However, the effect of fishmeal is much less with good (Gill *et al.*, 1987) than with poor quality silages (Gill and England, 1984). Girdler, Thomas and Chamberlain (unpublished observation, cited by Chamberlain *et al.*, 1989) investigated the mechanism of the protein-linked increase in food intake with cows in which fishmeal was given by dietary addition or by infusion into the abomasum (Table 1.7). Both dietary addition and intra-abomasal infusion of fishmeal significantly increased silage intake, suggesting the intake response can be mediated via an increased supply of protein to the abomasum. It seems that the absorbed products of protein digestion could contribute to intake control through a resetting of the digesta load at which 'fill' becomes a limiting factor (Gill *at al.*, 1988). Egan (1980) suggested that as the imbalance between energy and protein supply (ratio of protein to energy)

Table 1.7 The effect of protein supplementation via addition to the diet or via infusion into the abomasum on silage intake (After Chamberlain, Martin and Robertson, 1989)

	DM intake, kg/d	
Treatment	**Silage**	**Concentrate**
Basal diet (140g CP/kg DM)	7.5	6.0
Basal + 1.2 kg P^{+}	8.5	6.0
Basal + 0.6 kg P^{++} via abomasum	8.7	6.0

$^{+}$, a mixture containing (g/kg DM): 500 fishmeal, 300 bloodmeal and 200 meat and bone meal

$^{++}$, calculated to supply an equivalent amount of protein at the abomasum, assuming P has a rumen degradability of 0.5

becomes more adverse, intake is limited at a lower digesta load. Furthermore, protein supplementation may stimulate microbial activity in the rumen and produce a faster rate of digestion, leading to increased digestibility of the ration (Oldham, 1984). Therefore, it has been suggested that increased silage intakes in response to silage supplementation could arise from both ruminal and metabolic effects.

1.3.2 Effect of Protein Supplementation on Milk Production

Although the exact mechanism of the effects of protein supplementation on milk production is unclear, it has been suggested that the response could arise from increased microbial efficiencies due to increased rumen-degradable protein (RDP) supply resulting in increased flow of microbial protein to the small intestine or, from increased rumen-undegradable protein (UDP) supply from supplementary protein.

1.3.2.1 Ruminal Effects

Although microbial protein synthesis has often been increased by protein supplements, it is by no means a consistent observation. In some circumstances, even supplements of urea have increased the efficiency of microbial protein synthesis (Siddons *et al.*, 1979). Microbial protein synthesis can be improved by soya bean meal inclusion (Brett *et al.*, 1979; Rooke *et al.*, 1985) but the effect may depend on the level of inclusion of soya (Rooke, Alvarez and Armstrong, 1986). On the other hand, fishmeal inclusion did not increase the efficiency of microbial protein synthesis with diets of silage alone (Beever *et al.*, 1987) or silage and barley (Rooke and Armstrong, 1987). Rooke *et al.* (1987) observed an apparent synergism between intraruminal infusions of casein and glucose syrup on microbial protein synthesis (see Table 1.6), suggesting requirements for preformed amino acids and/or peptide. Supplements of sulphur and methionine have failed to increase the rate of protein synthesis although they have elicited a marked effect on the ruminal synthesis of microbial lipid (Chamberlain and Thomas, 1983).

1.3.2.2 Post-Ruminal Effects

It is possible that increased milk output and body weight gain elicited by protein supplementation (Castle and Watson, 1976; Rae *et al.*, 1986) could derive from an increase in UDP supply to the small intestine. As mentioned earlier, interpretation of responses of milk production to protein supplementation can be complicated by attendant increases in ME supply arising from increases in silage intake and of ration digestibility. However, Girdler, Thomas and Chamberlain (1988b) reported substantial milk production responses in the absence of effects on silage intake when cows received protein supplements containing fishmeal products. It seems that whatever the mechanism, these responses of milk production are apparently induced by an increased supply of UDP to the small intestine but to what extent they derive from direct effects of amino acids *per se* on milk synthesis or from indirect effects on voluntary intake and ration digestibility is not known.

Gordon (1979) obtained mean values of 0.36kg milk/% increase in concentrate CP with increasing the proportion of soya in the supplement and pointed out that responses to supplementary protein with silage remained linear up to a level beyond that observed with dried forage diets. However, in relation to the effects of different protein sources, Thomas and Rae (1988) examined a large data set and observed a considerable range in response to supplementary protein based on soya: 0-0.51kg milk/% increase in concentrate CP or 0-0.26g milk protein/g additional CP intake. With fishmeal based supplements, reports in the literature are conflicting. In an attempt to remove some of the variability due to differences in basal diet, Chamberlain *et al.* (1989) examined the effect of different protein sources in experiments with diets in which high-digestibility grass silage made up at least 60% of the total DM and concentrates were barley-based. Although milk production responses were variable, there was a general tendency for bigger responses to fishmeal than to soya. Mean increases in yield/increase in CP intake(g/g) of milk yield, milk protein and milk fat were 3.1, 0.12 and 0.08 for soya, and 4.1, 0.15 and 0.1 for fishmeal respectively. The patterns of response of milk production to level of fishmeal supplementation were also examined by Chamberlain *et al.* (1989) with cows were given *ad libitum* access to complete-mix diets. They concluded that the

pattern of response of milk fat output was particularly variable while the responses of milk protein output to increases in CP intake were reasonably close to linear, the average response being about 0.13g milk CP/g increased CP intake. Chamberlain *et al.* (1989) reasoned that the interpretation of the results of production trials in terms of protein supply to the small intestine is hindered by the fact that, for both fishmeal and soya, rumen degradability varies, not only with source but apparently, also with the level of inclusion in the diet (Gill and Beever, 1982; Rooke *et al.*, 1986; Rooke and Armstrong, 1987). Whether the bigger responses to fishmeal than to soya relate to in their UDP contents or to differences in their amino acid composition is not known. It appears that responses to fishmeal supplementation have been associated with a more efficient transfer of duodenal non-ammonia CP into milk protein than was the case for supplementation with soya (Oldham *et al.*, 1985) suggesting an effect linked to a superior amino acid balance of fishmeal. Soya and fishmeal differ in their content of essential amino acids, fishmeal being richer in lysine and especially methionine. However, where a fishmeal supplement induced a bigger milk production response than an equivalent amount of protein as soya, adding rumen-protected methionine and lysine to the soya-supplemented diet did not increase milk yield (Girdler, Thomas and Chamberlain, 1988a).

1.3.3 Amino Acid Nutrition

As mentioned above, interpretation of the response to dietary protein supplementation in terms of amino acid nutrition can be difficult but there is evidence that the post-ruminal supply of amino acids can limit milk output in some circumstances.

1.3.3.1 Effects of Infusion of Casein into the Abomasum

In his review of the literature Clark (1975) showed that there were responses in milk yield to intra-abomasal infusion of casein in cows receiving diets containing as much as 170g CP/kg DM; mean values were 7 and 12% increases for yields of milk and milk protein respectively. From an analysis of data from 28 experiments with cows given intra-abomasal infusions of casein, Thomas (1984) showed that milk protein content in animals given infusions

was raised on average by 1.5 g/kg as compared with untreated controls. This appeared to be an almost constant effect irrespective of the control milk protein level or the amount of casein given. Clark (1975) suggested that the response to casein could arise directly by supplying limiting amino acids, or indirectly, either by supplying carbon for gluconeogenesis or by altering hormone balance. Among these factors, the glucogenic effect is possibly of minor importance since abomasal glucose infusion did not show any milk production response (Ørskov, Grubb and Kay, 1977; Clark, 1975; König, Oldham and Parker, 1984). There is a discrepancy in reports of the possible effects of casein infusion on hormone balance. Whitelaw *et al.* (1986) reported that the concentration of growth hormone was decreased with increases in the level of casein infusion, whereas Oldham, Hart and Bines (1977) reported increases in growth hormone concentration with intra-abomasal infusion of casein. On the other hand, König *et al.* (1984) and Peel *et al.* (1982) failed to produce a change in blood growth hormone concentration when casein was infused into the abomasum. Such differences in response should be viewed against the known complexities of nutrient-endocrine interrelationships, which may question the meaning of simple descriptions of nutrient effects, or lack of them, on blood hormone concentrations. Ørskov *et al.* (1977) observed that for cows in early lactation and in negative energy balance the infusion of casein stimulated a mobilization of adipose tissue. In a subsequent study, Whitelaw *et al.* (1986) concluded that the primary response to casein is the correction of an amino acids deficit and that body fat mobilization is a secondary response to balance the higher ratio of amino acid-N:energy in the infused casein. They also noted that the milk yield response persisted until nitrogen equilibrium was achieved, after which the catabolism of excess protein was repartitioned towards the deposition of body tissue at the expense of milk energy secretion. There is also evidence that on silage-based diets abomasal infusion of casein stimulated milk production (Rogers, Bryant and McLeay, 1979).

1.3.3.2 Effects of Intravenous and Intra-Abomasal Infusion of Amino Acids and Supplementation of the Diet with Rumen-Protected Amino Acids

In many studies, responses to supplements of methionine and combinations of methionine and lysine given in rumen-protected form or as intravenous or intra-abomasal infusions have been inconsistent. For example, Rogers *et al.* (1979) reported an increase in milk protein yield of 13% in response to an abomasal infusion of methionine but, in contrast, Chamberlain and Thomas (1982) reported that an intravenous supplement of 8 g/d methionine increased milk fat yield and content by approximately 10% without effect on milk protein. Wong (1984) conducted a series of experiments to investigate the effect of intravenous infusions of methionine on milk production in which increasing levels of L-methionine were infused in cows given silage-based diets. Intraveneous infusions of methionine had a small or no effect on milk yield and the content of milk protein, but milk fat content and yield were increased. Girdler *et al.* (1988a and b) and Sloan and Thomas (unpublished observation, cited from Thomas and Thomas, 1989) reported that milk yield was not stimulated by protected methionine or a combination of methionine and lysine. In a review of a series of experiments to examine the response to methionine and lysine, Chamberlain *et al.* (1989) concluded that milk production responses have been limited to inconsistent responses in milk fat content sometimes, but not always, accompanied by increases in fat yield, and except in one experiment, supplementation of silage diets with methionine and lysine has been singularly unsuccessful in promoting increases in either milk yield or milk protein yield. Only in one experiment did methionine plus lysine supplementation increase milk protein concentration when 12g methionine plus 36g lysine was added to a diet containing about 185g CP/kg DM. The underlying mechanism of the milk fat response is not clearly established. However, indirect effects of methionine on microbial lipid synthesis in the rumen (Chamberlain and Thomas, 1983) as well as direct effects of methionine on milk fat synthesis in the mammary gland (Chamberlain and Thomas, 1982) are probably involved.

Thus, it has not been possible to replicate the milk production response to casein or protein supplements, such as fishmeal and soya, with supplements of one or two amino acids.

In this respect Girdler *et al.* (1988b) concluded that it required a complete mixture of amino acids to replicate the effect of protein supplements and that methionine and lysine alone would not suffice. Schwab, Satter and Clay (1976) demonstrated how milk production may be affected by the balance of amino acids infused (Figure 1.6). The responses in milk production to the infusion of a mixture of 10 essential amino acids were similar to those obtained from the infusion of casein. Infusion of lysine alone elicited a milk protein response equivalent of 16% of the total response in milk protein yield that was obtained with 10 essential amino acids and the corresponding value for lysine plus methionine was 43%. However infusion of arginine with histidine showed an increase in milk yield but not protein yield; these results suggest that there may be differential effects of individual amino acids on the secretion of both nitrogenous and non-nitrogenous constituents of milk.

1.3.3.2 Identification of Limiting Amino Acids

It is widely accepted that the mammary gland is the major site of nitrogen utilization in lactating ruminants (Mepham, 1982). The utilization of amino acids for milk protein synthesis in the mammary gland (Figure 1.7) has been studied with the use of the arterio-venous difference method (Bickerstaffe, Annison and Linzell, 1974; Mepham and Linzell, 1966; David, Bickerstaffe and Hart, 1978). One criterion often applied to identify potentially limiting amino acids at the mammary gland is that those amino acids in shortest supply relative to demand will undergo minimal metabolism within the gland. It would appear that on this basis, methionine, phenylalanine, tyrosine and tryptophan are potentially limiting. These amino acids apparently do not undergo net metabolism within the gland such that the amounts extracted from the arterial blood appear quantitatively in protein secreted in milk. On the other hand, valine oxidation and the incorporation of valine into aspartate, glutamate, glycine, alanine, isobutyrate and β-hydroxybutyrate have been reported (Mepham, 1982). Thus at the level of the mammary gland, methionine, phenylalanine, tyrosine and tryptophan are used most efficiently for milk protein synthesis. However, Oldham (1980) pointed out that while this suggests that they are potentially limiting, it has not been proved. In fact, at the level of the

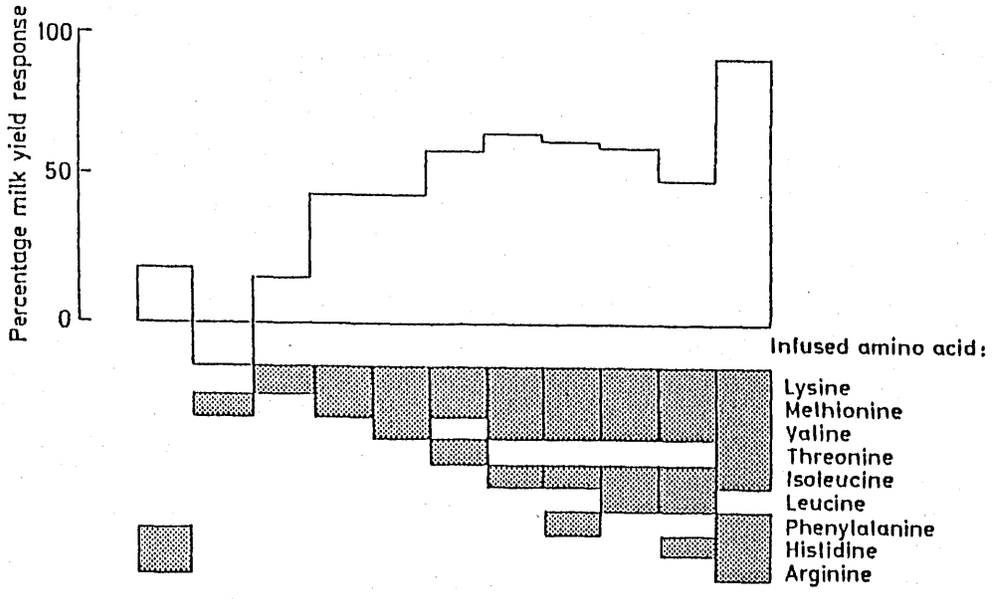


Figure 1.6 Milk yield response to infusion of various amino acid mixtures into the abomasum of cows. (After Schwab, Satten and Clay, 1976)

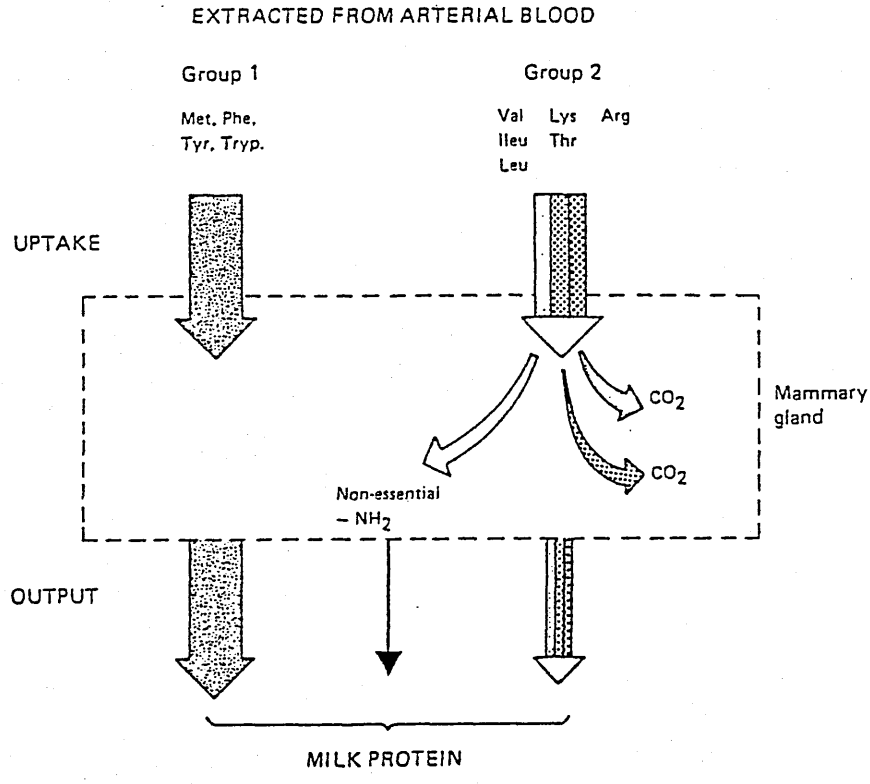


Figure 1.7 A simplified representation of essential amino acid (EAA) metabolism within the mammary gland. (After Mepham, 1979)

whole animal, lysine seems to be implicated as much as methionine and phenylalanine are. For instance, Chandler and Polan (1972) calculated the minimum transfer efficiencies for serum essential amino acid (EAA) and on this basis suggested that methionine, lysine, phenylalanine, tyrosine and threonine were the most limiting amino acids. Using the same approach, Vik-Mo, Emery and Huber (1974) reported that lysine and phenylalanine were the most limiting amino acids when cows were infused abomasally with either casein or glucose. Derrig, Clark and Davies (1974), using plasma amino acid concentrations as an indicator, observed that when casein was infused into the abomasum, threonine, phenylalanine and methionine showed the smallest increase relative to the amount infused in the form of casein, suggesting that they were the most limiting amino acids. Lysine, methionine and valine were most limiting when incremental amounts of formaldehyde-treated casein were added to a basal diet of 90g CP/kg DM to achieve rations containing 112, 135, 175 and 180g CP/kg DM (Broderick, Kowakzy and Sttter,1970). Schwab *et al.* (1976) concluded that lysine and methionine were the first two limiting amino acids for secretion of milk protein in cows receiving low protein-high maize diets. Oldham (1980) concluded that lysine may be first limiting when cows are fed maize-based diets, because the lysine content of duodenal digesta is lower in diets containing maize products than in diets without maize. Of course, the identification of limiting amino acids for milk production would be expected to be dependent on the dietary circumstances and physiological state of the cow.

Factorial approaches have also been used in an attempt to identify the amino acids which are most likely to limit milk production with grass silage diets. When sheep and cattle were offered silage, either alone or with barley, concentrations of methionine and lysine in duodenal digesta were low (Chamberlain *et al.*, 1986) compared to values obtained with hay-based diets (Thomas *et al.*, 1980d; Thomas and Chamberlain, 1982a), presumably reflecting the presence of a relatively low proportion of microbial protein since this is rich in methionine and lysine (Table 1.8). Thomas and Chamberlain (1982a) calculated the average composition of the mixture of indispensable amino acids absorbed (A), values for the amino acid composition of carcass tissue (T) and milk (M), using the values for the amino acid composition of duodenal

Table 1.8 The content (g/kg amino acids) of indispensable amino acids, histidine and arginine in duodenal digesta, grass silage, rumen bacteria, protozoa, pepsin and simulated duodenal digesta (After Thomas and Chamberlain, 1982a)

	Duodenal digesta	**Silage**	**Rumen bacteria**	**Rumen protozoa**	**Pepsin**	**Simulated duodenal digesta[+]**
Histidine	18	22	20	23	6	20
Threonine	58	56	59	54	81	59
Arginine	46	47	44	51	9	43
Methionine	18	16	25	22	15	20
Valine	66	68	63	54	60	64
Phenylalanine	54	55	57	66	56	57
Isoleucine	55	53	67	66	91	64
Leucine	88	84	83	92	90	85
Lysine	68	38	84	112	7	62

[+], calculated by assuming that 0.8 of the bacterial N was protein N

digesta given in Table 1.5 (Table 1.9). They speculated that methionine, lysine and threonine in the growing animal and methionine, lysine and valine in the lactating animal are the limiting amino acids for protein synthesis, though it should be stressed that such ratios must be interpreted with caution since they do not allow for differences between amino acids in their efficiency of utilization in the body tissues. Furthermore, Chamberlain *et al.* (1986) compared the calculated supply of essential amino acids to the duodenum and the estimated maximum output of EAA in milk with the observed output of EAA in milk in cows given a diet of silage and barley and concluded that methionine and lysine were limiting for milk production. Although methionine and lysine have been most strongly considered as first limiting amino acids for milk production with silage diets, results obtained by Girdler *et al.* (1988a and b) suggest that amino acids other than, or in addition to, methionine and lysine could be limiting milk production.

Table 1.9 The composition (g/kg total amino acids) of indispensable amino acids absorbed from the small intestine, deposited in body tissues and secreted in milk in animals given silage diets and the ratios of absorbed amino acids to tissue amino acids and milk amino acids (After Thomas and Chamberlain, 1982a)

	Amino acids absorbed from intestine (A)	**Amino acids in body tissue (T)**	**Ratio of A:T**	**Amino acids in milk (M)**	**Ratio of A:M**
Threonine	114	133	0.86	109	1.05
Methionine	49	65	0.75	60	0.81
Valine	142	129	1.1	155	0.92
Phenylalanine	139	113	1.22	116	1.2
Isoleucine	138	107	1.29	139	0.99
Leucine	235	230	1.02	229	1.03
Lysine	184	223	0.82	192	0.96

1.4 Aims and Objectives

A characteristic feature of grass silages is the rapid and extensive degradation of their nitrogenous constituents in the rumen and the relatively inefficient microbial capture of the ammonia released. The nutritional consequences are twofold. First, there is a reduced supply of amino acids to the small intestine and characteristically reduced concentrations of methionine and lysine in the amino acid mixture. Secondly, the host animal can be confronted with a greatly increased rate of ammonia absorption from the rumen which may lead to alterations of metabolism and nutrient utilization.

Further studies are required to establish the relative importance of these factors both for the control of the voluntary intake of silage and for its utilization for milk production.

The aims of the experiments in this thesis were:

(a) to investigate the effects of ammonia absorption from the rumen on voluntary intake and milk production

(b) to examine the effects of intra-abomasal infusions of different sources and levels of protein on silage intake and milk production

(c) to investigate the possible causes of different responses in silage intake and milk production to intra-abomasal infusions of different protein sources in relation to amino acid balance and the release of peptides during digestion.

CHAPTER 2

CHAPTER 2 MATERIALS AND METHODS

2.1 The Animals

2.1.1 Preparation of Surgically-Modified Animals

2.1.1.1 Rumen Cannulation

A solid nylon cannula of a type similar to that described by Jarret (1948) was used. The cannula was mushroom-shaped with a hollow stem, which was threaded on its external surface (48mm internal diameter and 20mm circular flange at the base). The flange was introduced into the rumen and the stem was exteriorized through the body wall and held in position by a nylon ring which was screwed down the stem of the cannula until it rested on the body wall. The cannula was closed with a screw cap.

The animal was fasted and denied access to water for 24 hours prior to the operation. Hair was removed from the area of operation by clipping and shaving. Immediately prior to the operation the animal was weighed and anaesthesia was introduced by intravenous injection of an appropriate amount of Rompun. Anaesthesia was maintained throughout the operation by the administration of halothane with a mixture of nitrous oxide and oxygen through a cuffed endotracheal tube. The animal was placed, on its right side, on an operating table and the area of operation was scrubbed with an antiseptic solution. For the insertion of the cannula a 12cm long incision was made with an electric cautery 12cm below the transverse processes of the lumbar vertebrae and 10cm posterior to the last rib of the cow. The muscle layers of the abdominal wall were separated along the direction of travel of their muscle fibres and retracted to expose the peritoneum which was incised to expose the rumen wall. A pouch of ventral rumen wall was exteriorized and punctured with a 10cm incision to allow the insertion of the cannula. Subsequently the base of the cannula was inserted through the incision and the wound closed to the stem of the cannula with a continuous suture. A second purse-string suture was made around the stem of the cannula and closed to prevent seepage of digesta. The cannula stem was then temporarily sealed with heavy swabs whilst a 4cm diameter flap of skin

was removed 10cm anterior to the initial incision. A scalpel-stab incision was made at this point and the subcutaneous tissue separated to allow the exteriorization of the cannula. The peritoneum and muscle layers were closed using continuous sutures and the skin was closed with single stay sutures. A nylon retaining ring was screwed into position over the stem of the cannula, the swabs removed and the cannula was sealed with a screw cap.

2.1.1.2 Abomasal Cannulation

The abomasal cannula consisted of a 1m or 1.5m length of surgical non-toxic, translucent vinyl tube of 5mm internal diameter (ID) and 8mm external diameter (ED: Portex, Hythe, Kent). The end was inserted into the abomasum and retained by a silicone rubber collar and washer (20mm ED).

The animals received an identical method of anaesthesia to that described above. An area of the right hand side, lower flank was shorn and washed prior to sterilization with disinfectant. A 15cm incision was made by cautery extending ventrally from a point 20cm posterior to the tip of the last rib of the cow. The abdominal wall and peritoneum were incised and the abomasum drawn back from its position behind the last rib. A 4cm incision was made laterally in the mid-lateral abomasal wall ensuring no digesta leaked into the body cavity. The cuff was inserted and secured with purse-string sutures in the mucosal lining and outer wall of the abomasum. The free end of the cannula was then secured to a 60cm long stainless steel needle through a 10 x 3mm eye. The needle was then manipulated upwards between the body wall and viscera and exteriorized between the last rib and transverse process of the right flank. At this stage a 1m length of 2mm ED vinyl tubing was inserted and passed down the cannula and secured at at its external end to reduce the risk of subsequent cannula blockage by the abomasal digesta. The incision was closed as described for the rumen cannulation.

2.1.1.3 Jugular Catheterization

To facilitate long term, stress-free blood sampling, an indwelling catheter of single lumen, medical grade polythene tubing of 1mm ID, 1.5mm ED (Dural Plastics, Australia) was

used. The catheter was passed through a previously inserted intravenous cannula (Medicut; Sherwood Industries, Argyll) until approximately 15cm lay in the jugular vein. An exteriorized portion of 20cm remained which was flushed with sterilized citrate-saline solution (0.5% w/v trisodium citrate, 0.9% w/v sodium chloride) and sealed by the partial insertion of a 20mm length of 1mm diameter stainless steel rod. Adhesive bandage was used to cover and secure the catheter.

2.1.2 Animal Management

2.1.2.1 Management of Surgically Prepared Animals

All animals were given an intramuscular injection of a broad spectrum antibiotic and the inside areas were dressed with antibacterial powder at the time of surgery.

Animals were allowed to recover from anaesthesia in a heated, padded room and usually did so within 1-2 hours of the operation. At this time they were offered hay and about 1 l water. Rations and water were reintroduced and on the day following the operation the animals frequently consumed their full rations of food. Complete recovery of appetite occasionally required a further 2-3 days. The skin stitches were removed 7-10 days after the operation and weekly removal of hair from around the rumen, and point of entry of abomasal cannulae together with cleaning, disinfection and antibiotic dusting (Acramide, Willington Medical, Shropshire) ensured the minimum of discomfort through insalubrity. Regular observation and disinfection of the area of exteriorization of the jugular catheter prevented jugular infection.

2.1.2.2 Management of Animals in the Experiments

The cows were housed individually in metabolism stalls with water and salt blocks freely accessible. In the urea infusion experiments, food was given in eight or six equal meal daily via a series of time feeders to stabilize the rumen conditions. Feed intake was determined daily prior to the afternoon milking and feeding was adjusted to ensure a feed refusal of approximately 15% of that offered. The animals were milked each day at 6.00 and 16.00 h. At regular intervals, a sample of foremilk was taken from each quarter for cell counts. Any

sign of subclinical infection in a particular quarter was treated with a an intramammary suspension of penicillin.

2.2 Experimental Techniques

2.2.1 Infusion Systems

2.2.1.1 Intraruminal Infusion

The multi-channel peristaltic pump (Watson and Marlowe 501M, Falmouth, Cornwall) with 98mm lengths of 2mm ID, 2.5mm ED silicone rubber tubing pumped urea solution from a reservoir, via polythene tubing, 1.5mm ID, 2.7mm ED, into the rumen. A bipartite infusion system was used for the infusion of urea and sodium bicarbonate into the rumen. Urea was infused by the means of multi-channel proportioning pump (Technicon, London) with 2.1mm ID silicone rubber tubing (Elkey products, Worcester). A second infusion pump (Watson and Marlow 501M) with a multi-channel peristaltic pump head infused sodium bicarbonate solution. The flow of bicarbonate infusion was controlled by 505D pH control module (LH Fermentation, Buckinghamshire) which, in conjunction with an indwelling liquid-proofed pH electrode (Russell pH, Fife) continuously monitored rumen pH and suspended the bicarbonate infusion at pH values above 7.0, recommencing infusion when pH fell below 6.5. The solution of bicarbonate was maintained at 40°C in a water bath to prevent crystallization. All infusion pipework and electrical connections to the pH probe entered the rumen via a pre-drilled rubber plug (Figure 2.1) held in place by the cannula screw cap. In the vicinity of the plug, in-line connectors were used. These were designed to pull open under the influence of any unusual strain on the infusion lines as would be produced, for example, if the lines became entangled with the animal.

2.2.1.2 Intra-Abomasal Infusion

The solutions and suspensions of protein sources were infused into the abomasum using a single-channel peristaltic pump (Watson and Marlow 502S) with 1.6mm ID silicon tubing (Belmont Instrument, Glasgow). The solution was continuously mixed throughout the infusion

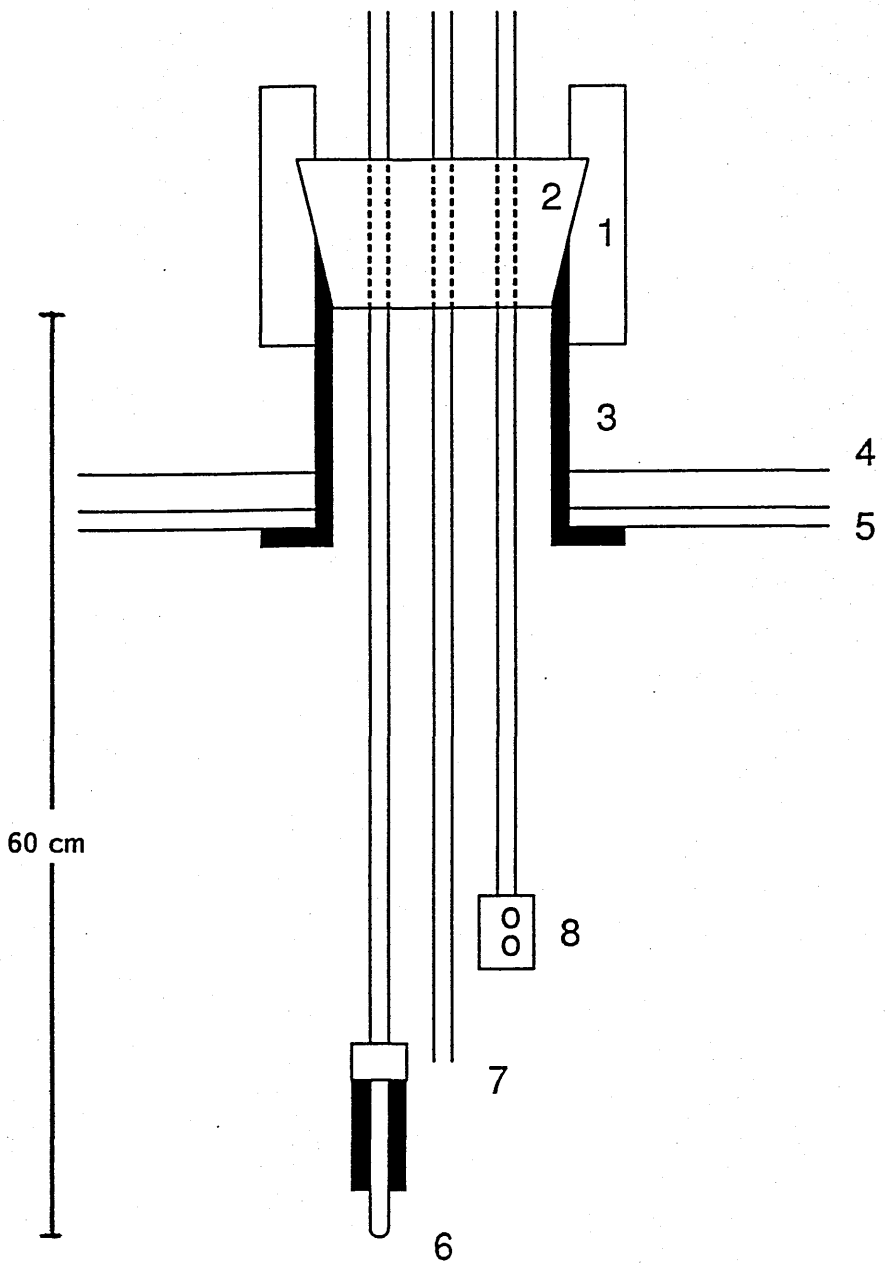


Figure 2.1 Diagrammatic representation of the cannula assembly for intraruminal infusion system. 1, screw cap; 2, drilled rubber plug; 3, rumen cannula; 4, body wall; 5, rumen wall; 6, water proofed pH probe; 7, sodium bicarbonate infusion line (3mm ID, 5mm OD); 8, protective cuff of urea infusion line.

period using a magnetic stirrer (FSA Laboratory Supplies, Leicester). An infusion period of 23 hours was allowed for all infusates and silicone rubber tubing at the peristaltic pump heads was replaced every 7 days regardless of apparent condition.

2.2.1.3 Preparation of Infusates

Urea (98%, Aldrich Chemical, Dorset) was dissolved in 4 l water. Sodium bicarbonate solution (10% w/w) was prepared by the gradual addition of feed grade sodium bicarbonate (Alkakarb, ICI) to a water at 70°C. Continuous mixing was used to facilitate complete dissolution and final volume was 4 l. Protein infusates were prepared by dissolving soluble casein for nutritional experiments (BDH Ltd, Dorset), casein enzymatic hydrolysates (Sigma Chemical, Dorset), Ardex SP6 soya protein isolate (British Arkady, Manchester) and untreated and heat-treated yeast products (Orsan Ltd, France) in 6 l water using an homogenizer (Siverson Machines, London). All protein infusates were freshly made up every day and stored at 4°C before use.

2.2.2 Dacron Bag Incubation Procedure

A Dacron bag technique was used to estimate the disappearance rate of dry matter and nitrogen of protein sources in the rumen (Mehrez and Ørskov, 1977).

Dacron bags of approximately 20 x 8cm with a mesh size of 45μm with double stitched seams and curved corners, were used to hold a sample *in situ* in the rumen of fistulated cows given silage *ad libitum*. A known fresh weight (about 5g) of soya bean meal and fishmeal was placed in the bags. They were then tied at the neck and attached by a length of nylon string to a ring which in turn was tied to the cap of the rumen cannula. The total length of string was around 55cm. At the end of each incubation interval (0, 8, 16, 24 and 48 hours) the bags were removed and washed thoroughly with tap water. After drying the bags, the rate of DM disappearance was calculated and residues were used for determination of nitrogen content to calculate the disappearance of nitrogen. The DM and N disappearance rates of animal protein (AP) in Experiment 1a in Chapter 4 are shown in Table 2.1.

Table 2.1 Dry matter and nitrogen disappearance (%) of animal protein (AP) after incubation in the rumen in Dacron bags (values are mean of six replications with standard error)

	Rumen incubation time, h				
	0	8	16	24	48
Dry matter	13.4 ± 1.0	24.4 ± 1.4	28.1 ± 1.1	31.6 ± 0.5	36.7 ± 1.0
Nitrogen	11.4 ± 1.0	20.9 ± 1.5	25.2 ± 1.5	31.8 ± 0.5	40.6 ± 0.7

2.3 Collection and Preparation of Samples

2.3.1 Feedstuffs

Silage samples were taken from the silo at least once during each treatment period, from which 1500g were dried and ground through a 1mm screen for dry sample analysis. Another 1500g was minced through a 100mm dye (Crypto Ltd, London) and stored frozen for analysis.

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

2.3.2 Digesta

Samples of rumen liquor were obtained under suction by inserting a 30cm long metal tube, along the length of which a series of holes had been bored, through the rumen cannula into the posterior ventral sac of the rumen. The pH of the rumen liquor was determined (Corning 120 pH meter; Corning Ltd, Staffordshire) immediately after collecting the sample and a large particles of food material were removed by squeezing the digest through a double layer of muslin. Five ml of the filtrate was preserved in tungstic acid for ammonia nitrogen determination. The digesta were then centrifuged at 1500g for 20 minutes and the supernatant stored at -20°C prior to analysis.

2.3.3 Blood

Samples of blood were withdrawn from either a jugular vein or tail artery. For the jugular sample, blood was taken via an indwelling polythene cannula inserted a minimum 18 hours before sampling commenced. Patency of the cannula was maintained by flushing and filling it with a sterile solution of 0.9% (w/v) sodium chloride and 0.5% (w/v) trisodium citrate between sampling. Blood samples were taken into syringes that had been rinsed with heparin solution (250 units/ml; Evans Medical Ltd, Middlesex) and dried before use. The heparinized blood was transferred to heparin-treated centrifuge tubes placed in ice.

The tail blood samples were withdrawn directly from the tail artery into lithium

heparin-teated vacutainers (Becton and Dickinson, Wembley) through a small bore (20G) needle while animals were restrained in their stalls. Samples were centrifuged at 1500g for 15 minutes and the blood plasma was removed. Plasma was stored immediately at -20°C before use.

2.3.4 Milk

Milk samples were collected from the last four consecutive milkings of each treatment period. Approximately 300ml of milk were collected into bottles containing 280mg potassium dichromate (Thompson and Capper Ltd, Cheshire), mixed thoroughly to dissolve the preservative and stored at 4°C. Samples to be bulked were warmed slowly to 40°C, the fat globules were dispersed by gentle shaking and subsamples were combined in proportion to the respective milk yield. A 50ml aliquot was stored at -20°C for analysis of milk fatty acid composition and the remainder was stored at 4°C.

2.4 Analytical Methods

2.4.1 Dry Matter and Ash

Dry matter and ash contents in all samples of feedstuffs with the exception of silage, were determined by standard methods. A known weight of sample was oven dried at 100°C to constant weight and the dry matter expressed as a percentage of fresh weight.

The Dean and Stark dry matter content was determined by distillation of a minced silage sample with toluene following the procedure of Dewar and DcDonald (1961).

Reagents

1) Toluene, distilled (110-120°C) to remove any water
2) Neutral ethanol (25ml ethanol was titrated against 0.1M NaOH and then a stock solution prepared according to the values obtained)

Procedure: Twenty-five g of minced silage were placed in a round-bottomed 1 litre flask and immediately covered with 300ml of redistilled toluene. The refluxing was continued until the level of water in the receiver did not change over a period of 15 minutes. The water receiver

was then disconnected and left for 1 hour, after which the volume of water was read and toluene removed using a Pasteur pipette. Ten ml of water was pipetted into a 25ml volumetric flask and diluted to volume. The acidity of the water was measured using 10ml of the diluted liquid. Forty ml of neutral ethanol was added and titrated with 0.1M NaOH using phenolphthalein as a indicator.

Calculation

Volume correction = 2.5 x V/10 x T x 0.0055 ml =0.001375VT ml

At 20°C wt of water = 0.998V (1 - 0.01375T)

wt of silage DM = W -0.998 (1 - 0.001375T)

% of Dry matter = 100 X{W - 0.998V (1 - 0.001375T)} / W

where W = weight of fresh silage (g)

V = observed volume of water (ml)

T = titre of 0.1M NaOH (ml)

Ash content was determined by ignition of a known weight of dry matter in a muffle furnace at 550°C for at least 3 hours, and expressed as a percentage of the dry sample.

2.4.2 pH of Silage

A representative sample of 20g of wet silage was taken and mixed in 20ml of distilled water and the pH was read using a Corning 120 pH meter.

2.4.3 Total Nitrogen

The nitrogen content of feed samples was measured by a macro-Kjeldahl method using the Kjeltec apparatus (Tecator Ltd, Bristol). A sample containing 1-2mg nitrogen was digested (420°C) with 98% (w/v) sulphuric acid (nitrogen free) and catalyst tablets containing 2g potassium sulphate and 0.02g selenium. The digested sample was distilled with sodium hydroxide and distillate (150ml) was collected into a conical flask containing 25ml boric acid solution (40g/l). Nitrogen was determined by titration of ammonia with 0.01M HCl (0.04M HCl for silage samples) after the addition of ethylene blue/methylene red indicator solution.

Crude protein content was calculated by multiplying the total nitrogen content of the sample by 6.25.

2.4.4 True Protein and Non-Protein Nitrogen (NPN) in Silage

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

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

Procedure: Wet minced silage (1g) was weighed accurately into a centrifuge tube and 20ml 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 1500g 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 25ml 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 as described previously.

2.4.5 Ammonia Nitrogen in Silage

This was determined on a water extract of the sample. The extract was prepared by placing 20g wet, minced silage and 200ml 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 was centrifuged at 1500g for 20 minutes and the supernatant was retained and stored at -20°C.

Procedure: Ten ml of 10M NaOH was added to 10ml of silage extract and the ammonia was distilled over into 25ml boric acid solution (40 g/l) using the Kjeltec apparatus and the distillate was titrated with 0.01M HCl, as described for the determination of total nitrogen.

2.4.6 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 0.5M sulphuric acid.
2) 0.05M iodine solution, prepared by dissolving 20g potassium iodide in 35ml water and then dissolving 13g of iodine in this solution and made up to 1 l with water.

Procedure: 9.5ml silage extract were transferred into a 15ml centrifuge tube and 0.5ml copper sulphate and 1g 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 100ml flask with 0.5ml of 5M sulphuric acid and a few anti-bumping granules and steam distillation was commenced, 5ml ceric sulphate being added through separating funnel. Fifteen ml distillate was collected in a 50ml conical flask containing 2ml of 0.5% (w/v) sodium metabisulphate. One ml of 2% (w/v) starch solution and 0.05M iodine were added until a permanent blue colour was obtained and then decolourization was achieved by adding 5mM iodine until the pale blue colour persisted. This was titrated with 5mM iodine after adding 1g sodium hydrogen carbonate.

2.4.7 Total Soluble Sugars in Silage

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

Reagents

1) Somogyi reagent, prepared by dissolving 28g anhydrous di-sodium hydrogen

orthophosphate and 40g potassium sodium tartrate in 700ml stilled water; then 100ml of 1M NaOH, 80ml of 10% (w/v) hydrated copper sulphate and 180g anhydrous sodium sulphate were added, and the volume made up to 1 l.

2) Arsenomolybdate reagent, prepared by dissolving 25g ammonium molybdate in 450ml water then adding 21ml Analar concentrated sulphuric acid. A solution of 3g (12%) di-sodium hydrogen arsenate in 25ml 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.

3) Reagent A, B and C

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

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

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

Procedure: A sample (5ml) of silage extract was pipetted into a glass stoppered test tube for hydrolysis. 0.1ml of 1M 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 0.1ml of 1M NaOH was added. Two ml of hydrolysate were transferred to a 15ml centrifuge tube and deproteinized by adding 4ml of 5% (w/v) zinc sulphate solution and 4ml of 0.3M sodium hydroxide. After mixing the tube and its contents were centrifuged at 1500 g for 10 minutes. Two ml of supernatant or standard were transferred to a glass stoppered test tube containing 2ml of reagent C. The tube was heated in a boiling water bath for 10 minutes. After cooling, 2ml of arsenomolybdate reagent were added, the solution transferred to a 50ml volumetric flask and made up to volume with water. The absorbance was read on a Lambda 5 spectrophotometer (Perkin Elmer, Buckinghamshire) at 500nm against a blank of distilled water. The total soluble sugars in samples were calculated by reference to a calibration graph derived with standard solutions of D-glucose containing 50 to 250 mg/l.

2.4.8 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 5ml of silage extract and 1μl injected on to the column of the 8310 gas chromatograph (Perkin-Elmer, Buckinghamshire) fitted with a flame ionization detector. The columns were 2m long and of 2mm 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.

2.4.9 Total and Individual Volatile Fatty Acids (VFA)

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

Reagents

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

Procedure: Two ml of silage extract were transferred to a 10ml test tube and 1ml preservative and 2ml hexanoic acid were added and the contents mixed well. The tube was shaken and allowed to stand for 20 minutes and then centrifuged at 1500g for 20 minutes. The supernatant was analysed using a Shimadzu GC-8A gas chromatograph (Dyson Instruments Ltd, Tyne and Wear). The sample (1-3μl) was injected onto a glass column packed with 5% Carbowax 20M/TPA on Chromosorb G 80/100 mesh. The oven temperature was 135°C and carrier gas (N_2) flow rate was 60 ml/min.

The molar concentration was calculated for each acid from the peak area on the chromatograph relative to that of hexanoic acid. Corrections were made for the differences in

the response of the detector to each acid using factors derived from the analysis of a standard VFA solution.

2.4.10 Neutral Detergent Fibre (NDF) and Acid Detergent Fibre (ADF)

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

Reagents

1) Neutral detergent (ND) solution contained 30g sodium lauryl sulphate, 18.6g disodium ethylenediaminetetraacetic acid dihydrate, 6.81g sidium borate decahydrate, 4.56g anhydrous di-sodium hydrogen phosphate and 10ml 2-ethoxyethanol in 1 l of solution.
2) Acid detergent (AD) solution consisted of 20g cetyl-trimethyl ammonium bromide per l of 0.5M sulphuric acid.

Procedure: Approximately 1g of sample was added to a 500ml round bottom flask to which 100ml of ND solution, 2ml Dekalin and 0.5g sodium sulphite for NDF determination or 100ml AD solution and 2ml 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.

2.4.11 Determination of Digestibility *in vivo*

Silage was offered at approximately 1.2 x maintenance level of feeding to three or four mature wether sheep held in metabolism cages designed for the separate collection of faeces and urine. When the sheep were established on the appropriate silage they were fed at a constant level of intake for a period of 21 days. Complete faecal collections were made over

the last 7 days of this period. Digestibility was recorded as the concentration of digestible organic matter in the dry matter (DOMD).

2.4.12 Determination of Individual Amino Acids

The amino acid composition of feeds, infusates and blood plasma was determined by the modified method of Umagat, Kucera and Wen (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 amino acid, 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 used consisted of a Spectra-Physics Model SP-8700 solvent delivery system (Anachem Ltd, Luton, Bedfordshire) coupled to a Gilson Model 121 filter fluorometer (Anachem Ltd) with wavelength of 305-309nm excitation filter and a 430-470nm emission filter. Separations were carried out on a 250 x 4.6mm ID Apex II column prepacked with 5μm octadecyl particles (Johns Chromatography, Hengoed, Mid Glamorgan) connected to a pre-column (10 x 4.6mm ID). Sample injections were made using Gilson Model 401 sample dilutor and 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 125mg OPA with 2.5ml HPLC grade methanol in a 25ml volumetric flask and then made up to volume with 0.4M sodium borate buffer (pH 9.5). 100μl MCE was added to the solution and stored in dark and allowed to stand for 24 hours before use. Every day 10μl of MCE were added.
2) 0.4M sodium borate buffer (pH 9.5), made by dissolving 24.732g boric acid in 970ml water and pH adjusted to 9.5 with 4M NaOH. The solution was made up to 1l with water

and filtered through 0.47μm pore size filter (Supelco, USA).

3) 6M HCl solution, prepared by adding 501ml concentrated HCl (specific gravity 1.18) to 499ml water and then 0.5ml of MCE was added.

4) Citrate buffer (pH 2.2), prepared by dissolving 19.6g sodium citrate in 700ml water and adding 16.5ml concentrate HCl, 20ml thiodiglycol, 2ml Brij-35 solution and 0.1ml octanoic acid and made up to 1l with water.

5) Solvent A consisted of 0.05M sodium acetate (pH 5.8), HPLC grade tetrahydrofuran and HPLC grade acetonitrile in a ratio of 96:1:3.

6) Solvent B, comprised HPLC grade methanol.

7) Solvent C consisted of 1% (v/v) methanol.

8) Amino acid standard, prepared by adding asparagine, glutamine, ornithine, taurine, α-aminobutyric acid, γ-aminobutyric acid, tryptophan and 3-methylhistidine solution to the commercial Sigma standard (AA-S-18) for plasma analysis. The commercial Sigma standard was used for feed and infusate analysis. The concentration of standard was 500 nmol/l and the standard was kept at -20°C until analysed. For feed analysis, standard was diluted to the concentration of 33.3nM with citrate buffer and with 0.01M HCl for plasma analysis.

9) Internal standard (500nM), prepared by dissolving 0.0234g ethanolamine in 100ml water and stored at 4°C. For feed analysis, this was diluted to a concentration of 33.3nM with citrate buffer and with 0.01M HCl for plasma analysis.

Preparation of samples

Feeds and Infusates

Feed and infusate samples were prepared using acid hydrolysis. The sample containing approximately 15mg total nitrogen was transferred into 80ml Quickfit tube, 70ml of 6M HCl were added and the tube stoppered. The flask was maintained for 24 hours at 105°C and then cooled to 4°C. The contents were filtered through Whatman No.42 filter paper into a 200ml volumetric flask, and washed and made up to volume with water. One ml of filtrate was diluted with 1ml of 2M NaOH and 8ml water, and 2ml of internal standard was added to 2ml

of dilutant. This mixture was filtered through a 0.2μm syringe filter (Gelman, Northampton).

Plasma

Plasma samples were deprotenized 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 1500g. One ml of supernatant was diluted with 0.01M HCl, and 2ml of internal standard were added to the solution. This mixture was filtered through a 0.2μm syringe filter.

Chromatographic Conditions: OPA/MCE derivatization was conducted by dilutor at room temperature and 20μl of the derivatized mixture was injected by automatic sample injector. The solvents used were degased with helium throughout the analysis and the gradient program was applied (Table 2.2). The flow rate was maintained at 1ml/min. The sensitivity of the fluorimeter was set at 0.5 to reduced the base line noise and the attenuation of integrator was set at 3.34. Analytical run time was 42 minutes.

Procedure: The amino acids in the hydrolysate and deprotenized plasma were separated by elution with solvents in order of hydrophobicity. A schematic diagram of the HPLC system is given in Figure 2.2. The OPA/MCE reagent vial, a 100μl standard or sample vial and a mixing vial were placed on the sample rack in the automatic sample injector. A few minutes after starting the gradient program, the analysis program was memorized through the control panel using the following commands: tubing volume = 220, air gap volume = 2, sample no. = n, sample volume = 30, injection volume = 20, needle flush volume = 500, injection valve rinse volume = 500. Identification of the individual amino acids was made by reference to their retention times measured under the given conditions when a standard mixture of amino acids was analysed. A typical chromatogram of an amino acid standard, a plasma sample and an infusate are shown in Figures 2.3, 2.4, 2.5 and 2.6. The reproducibility of the standards and the determination of the amino acid compositions of soluble casein and a plasma sample in 5 runs are given in Table 2.3 and Table 2.4.

Table 2.2 Chromatographic gradient conditions for HPLC analysis

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	100	0	1
50	100	0	1

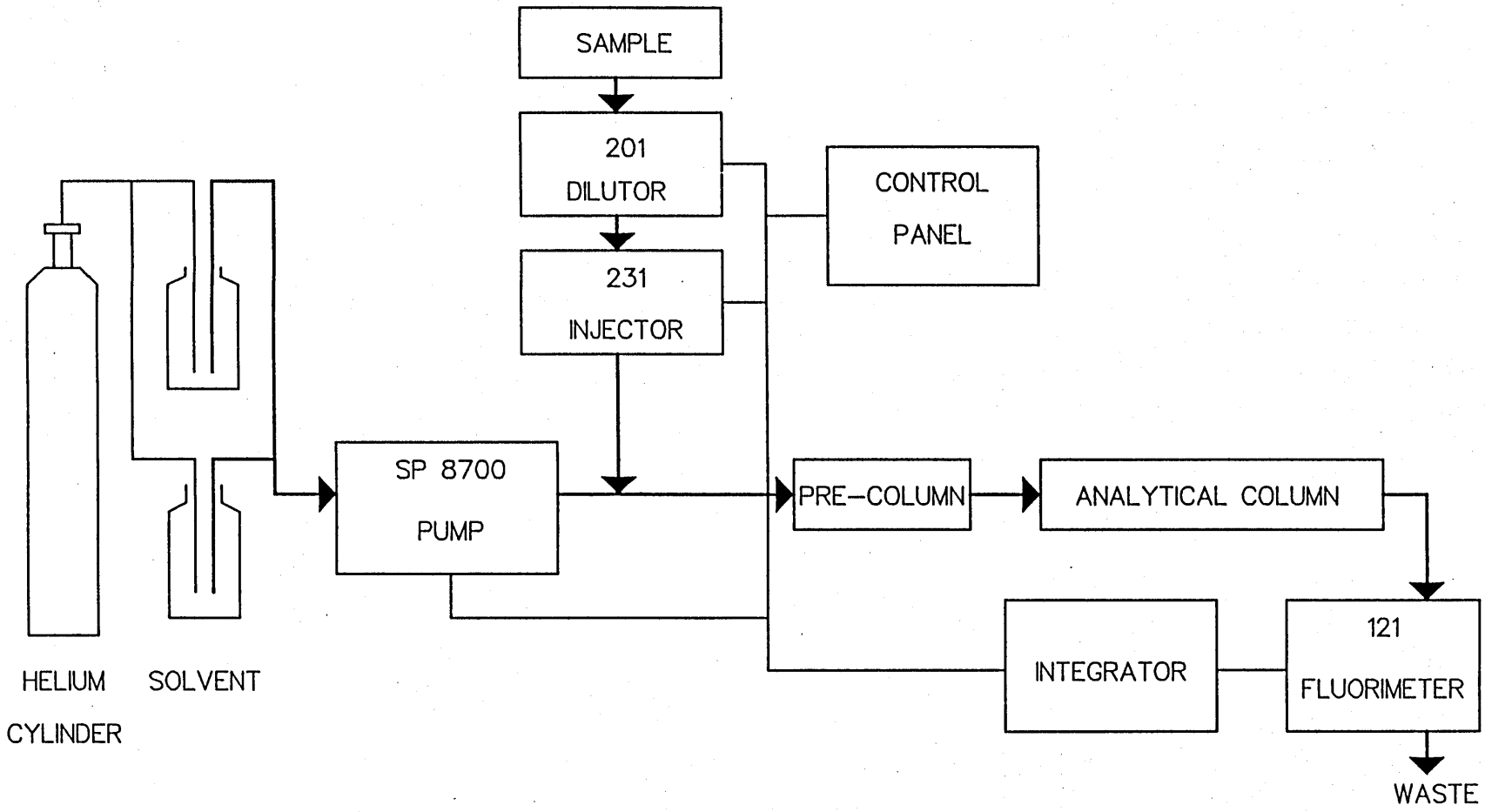
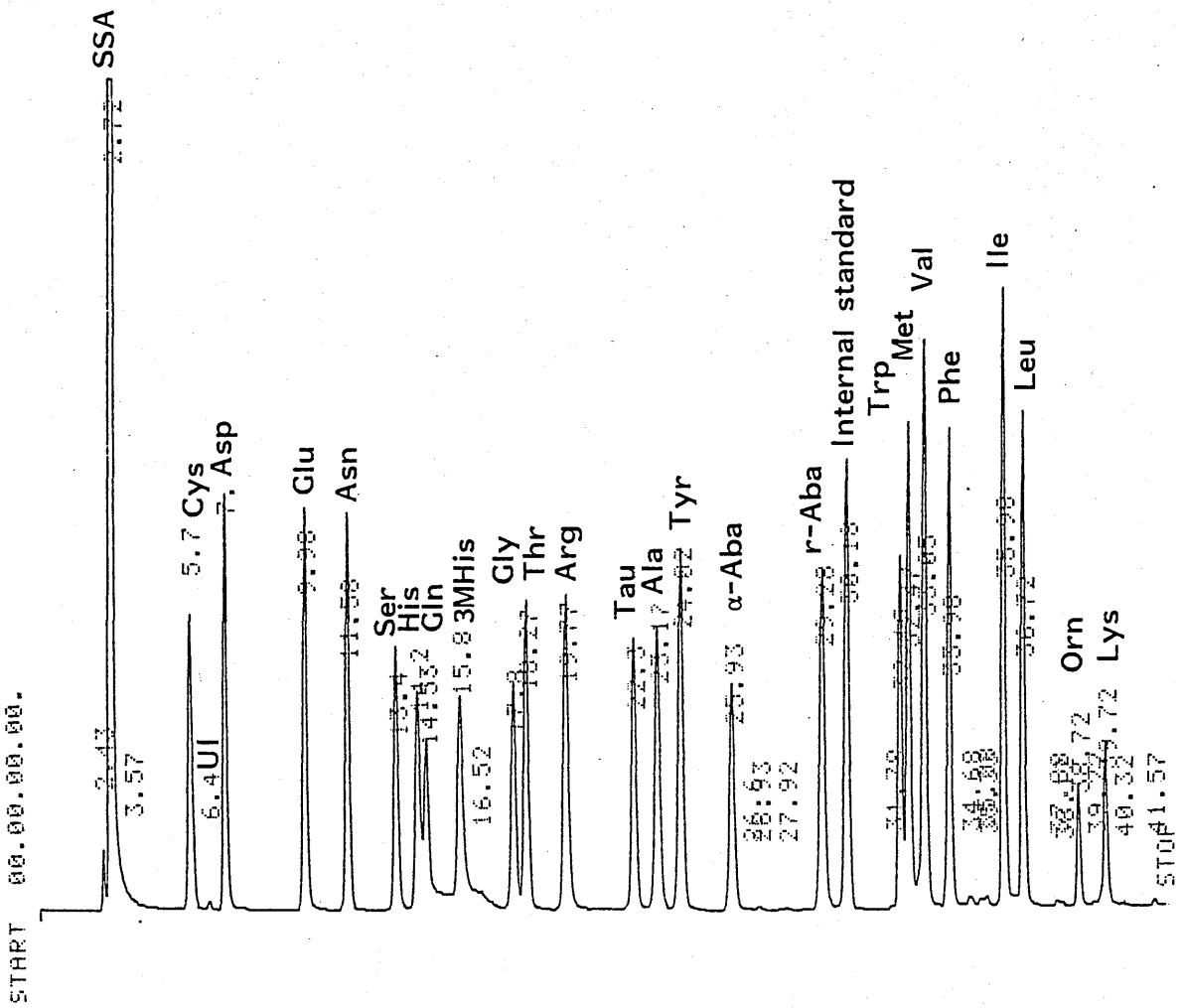


Figure 2.2 Schematic diagram of HPLC system



igure 2.3 Chromatogram of an amino acid standard for plasma analysis Cys: Cysteic acid, Asp: Aspartic acid, Glu: Glutamic acid, Asn: Asparagine, Ser: Serine, His: Histidine, Gln: Glutamine, 3MHis: 3-methyl histidine, Gly: Glycine, Thr: Threonine, Arg: Arginine, Tau: Taurine, Ala: Alanine, Tyr: Tyrosine, α-Aba: α-Aminobutyric acid, r-Aba: r-Aminobutyric acid, Trp: Tryptophan, Met: Methionine, Phe: Phenylalanine, Val: Valine, Ile: Isoleucine, Leu: Leucine, Orn: Ornithine, Lys: Lysine, UI: Unknown impurity.

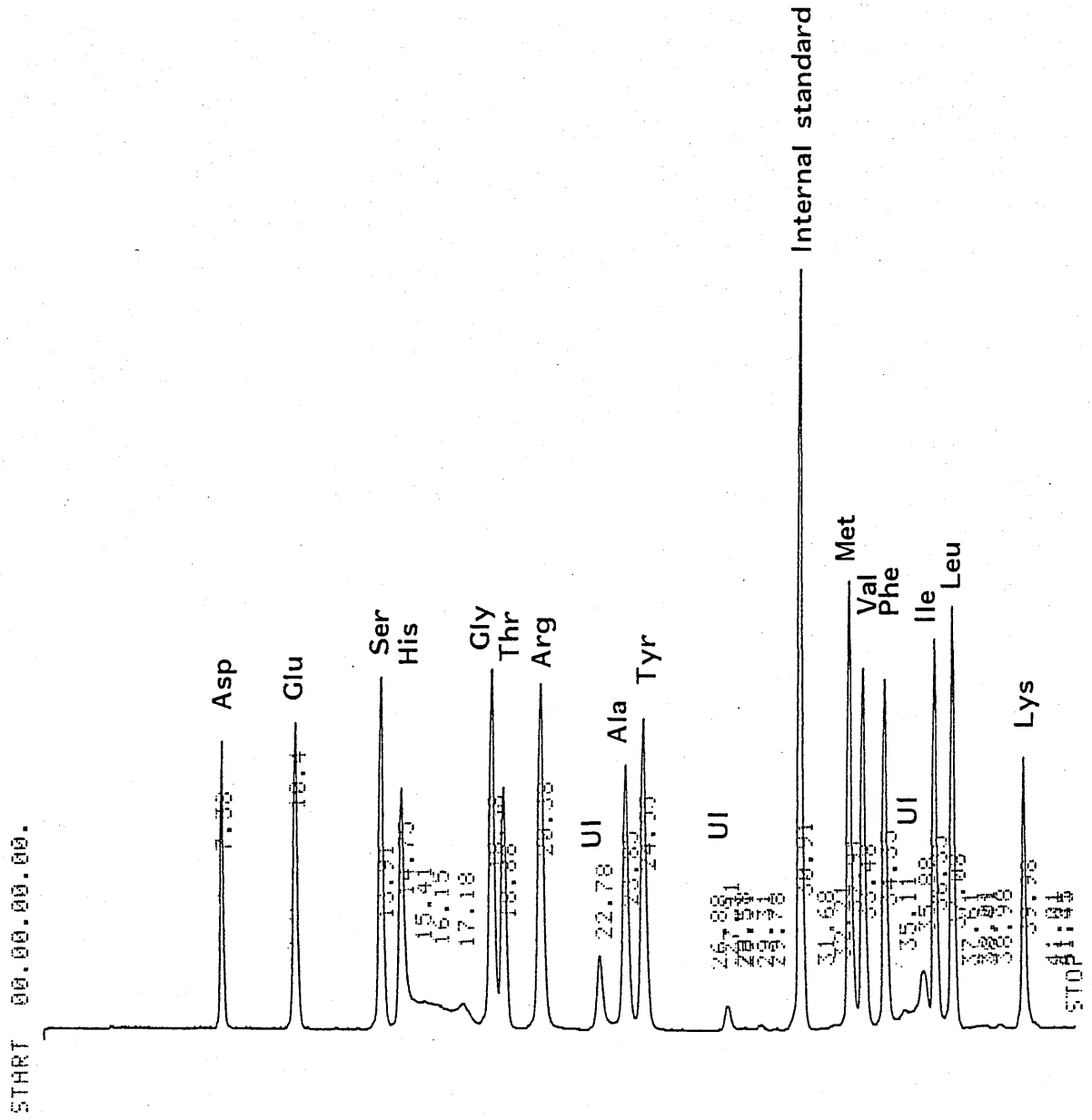


Figure 2.4 Chromatogram of an amino acid standard for feed and infusate analysis
UI; Unknown impurity

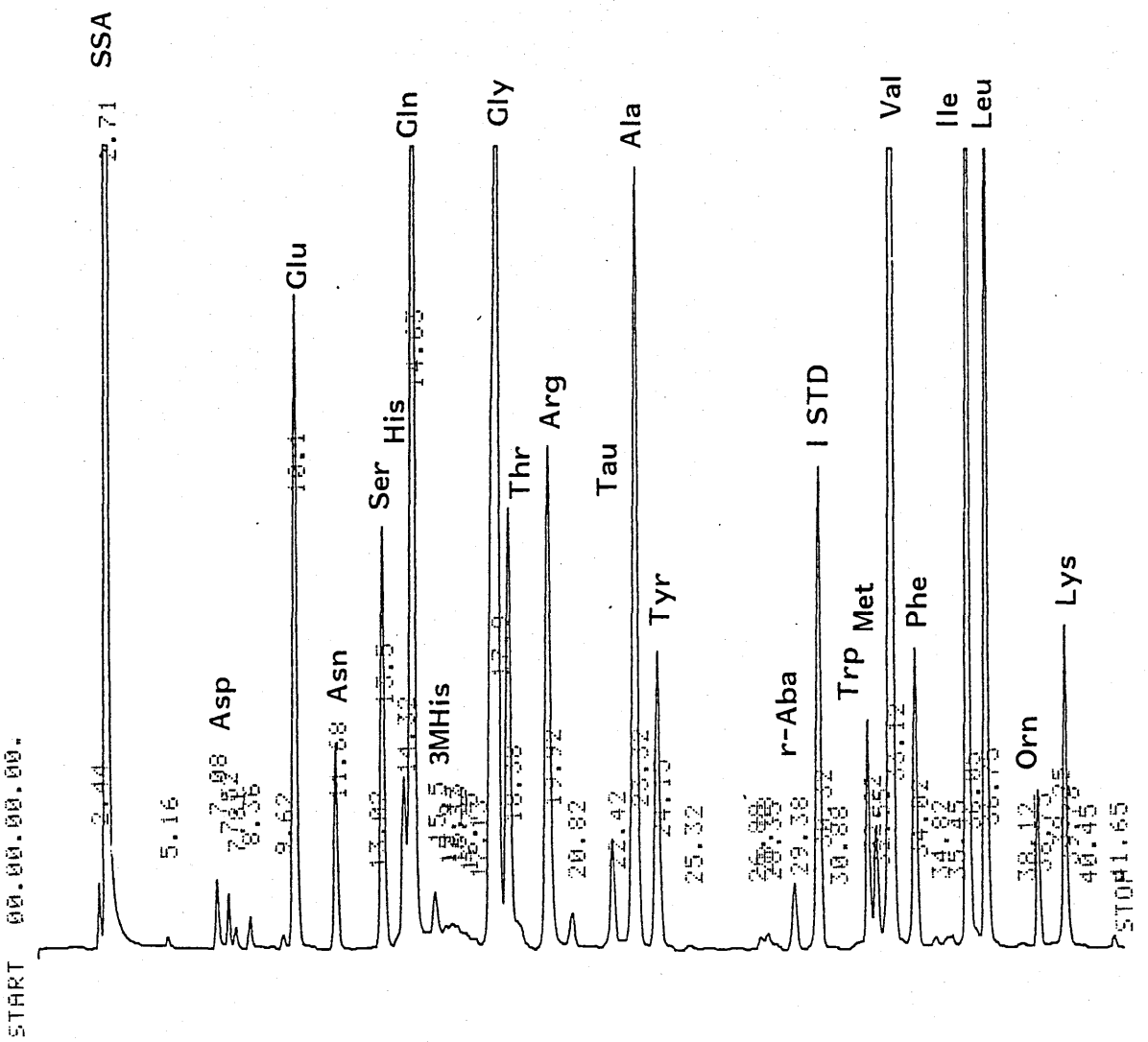


Figure 2.5 Typical chromatogram of plasma sample

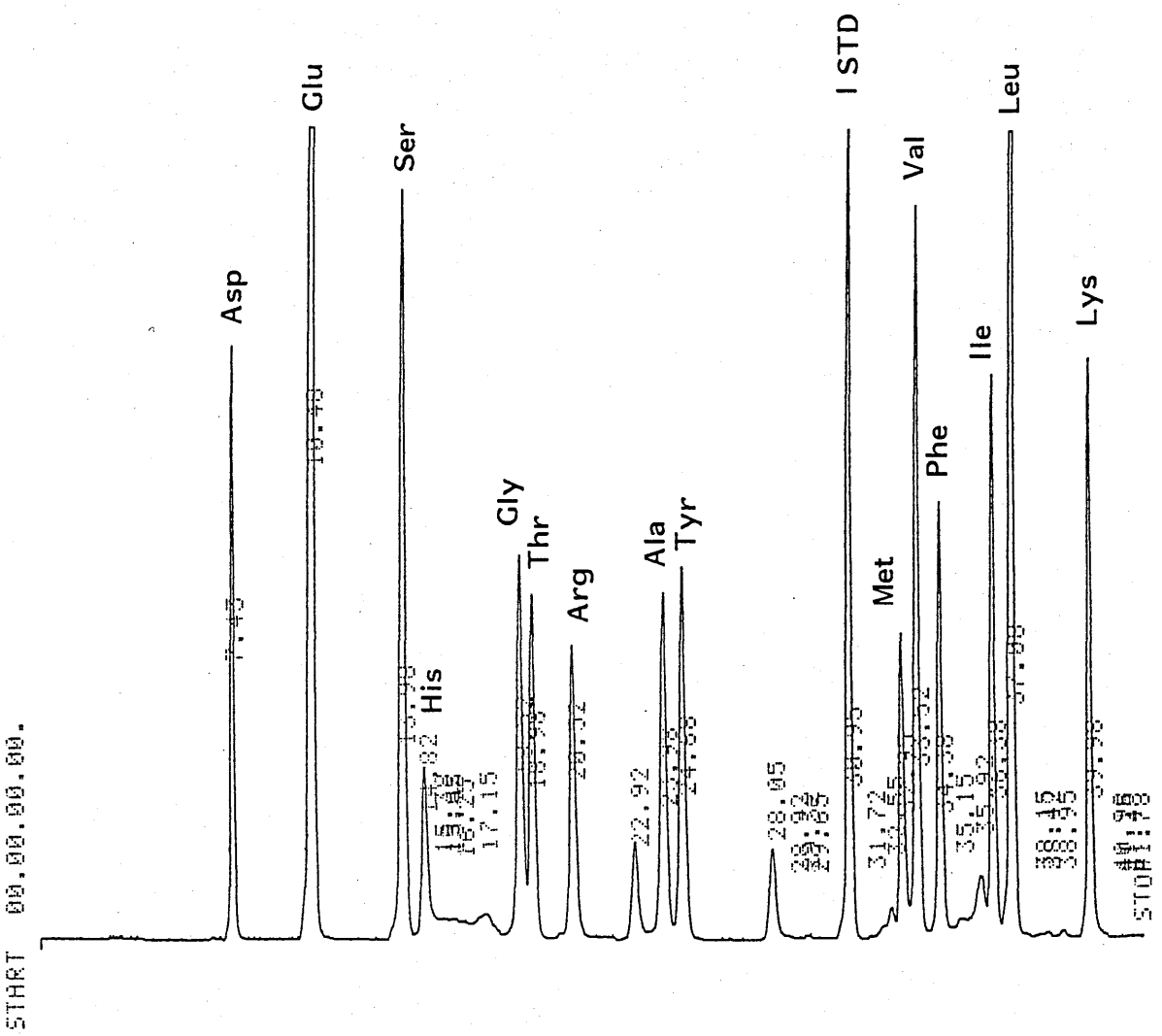


Figure 2.6 Chromatogram of an acid hydrolysate of soluble casein

Table 2.3 The relative areas of individual amino acids in the standards relative to the internal standard ethanolamine (values are mean of nine replications with standard error)

	Standard for plasma	**Standard for feed and infusate**
Cysteic acid	64.77 ± 0.61	nd[+]
Aspartic acid	71.26 ± 0.43	42.01 ± 0.48
Glutamic acid	78.99 ± 0.41	54.75 ± 0.42
Asparagine	75.67 ± 0.38	nd
Serine	54.44 ± 0.28	59.07 ± 0.24
Histidine	50.03 ± 0.33	55.59 ± 0.74
Glutamine	37.18 ± 0.54	nd
3-Methylhistidine	57.52 ± 1.19	nd
Glycine	52.14 ± 0.19	60.95 ± 0.51
Threonine	69.18 ± 0.28	51.78 ± 0.37
Arginine	79.51 ± 0.24	72.18 ± 0.56
Taurine	61.73 ± 0.39	nd
Alanine	71.16 ± 0.50	59.43 ± 0.30
Tyrosine	86.28 ± 0.57	65.78 ± 0.51
γ-Aminobutyric acid	54.78 ± 0.27	nd
α-Aminobutyric acid	85.51 ± 0.50	nd
Tryptophan	67.39 ± 0.49	nd
Methionine	92.90 ± 0.51	75.00 ± 0.20
Valine	117.32 ± 0.97	76.22 ± 0.72
Phenylalanine	90.80 ± 0.82	62.18 ± 0.40
Isoleucine	115.89 ± 1.05	74.41 ± 0.76
Leucine	93.21 ± 0.76	70.37 ± 0.53
Ornithine	22.5 ± 0.45	nd
Lysine	34.82 ± 0.96	32.56 ± 0.39

[+]nd, not determined

Table 2.4 The reproducibility of the determined amino acid contents in a sample of plasma and soluble casein (values are mean of five replications with standard error)

	Plasma (μmol/l)	**Casein (g/100g CP)**
Aspartic acid	10.96 ± 0.48	7.43 ± 0.15
Glutamic acid	102.75 ± 0.51	15.70 ± 0.12
Asparagine	31.90 ± 0.84	nd
Serine	98.26 ± 0.37	6.91 ± 0.03
Histidine	49.89 ± 0.70	3.13 ± 0.04
Glutamine	842.88 ± 8.41	nd
3-Methylhistidine	21.57 ± 0.58	nd
Glycine	415.55 ± 1.68	2.35 ± 0.05
Threonine	103.69 ± 0.28	4.71 ± 0.07
Arginine	104.22 ± 0.55	4.09 ± 0.06
Taurine	27.00 ± 0.51	nd
Alanine	172.08 ± 1.31	3.32 ± 0.02
Tyrosine	51.32 ± 0.38	5.92 ± 0.10
α-Aminobutyric acid	11.73 ± 0.08	nd
Tryptophan	44.67 ± 0.38	nd
Methionine	17.18 ± 0.16	3.21 ± 0.04
Valine	155.46 ± 3.75	6.13 ± 0.10
Phenylalanine	43.95 ± 0.26	5.59 ± 0.06
Isoleucine	112.28 ± 1.38	5.55 ± 0.18
Leucine	107.82 ± 0.32	8.61 ± 0.11
Ornithine	83.04 ± 0.96	nd
Lysine	119.53 ± 2.12	9.13 ± 0.09

2.4.13 Ammonia Nitrogen in Rumen Liquor

Ammonia nitrogen in rumen fluid was determined by a colorimetric technique using phenol-hypochlorite.

Reagents

1) Tungstic acid reagent consisted of 70ml of .5M sulphuric acid, 10% (w/v) sodium tungstate, 0.1ml orthophosphoric acid and 800ml water.
2) Reagent A was prepared by dissolving 13g NaOH and 4g sodium ethylenediaminetetraacetic acid in 1 l water.
3) Reagent B was prepared by dissolving 10g phenol and 0.05g sodium nitroprosside in 1 l water.
4) Reagent C was prepared by dissolving 5g NaOH, 31.8g trisodium phosphate and 10g sodium hypochlorite in 1 l water.

Procedure: A sample of rumen liquor (5ml) was transferred to a 50ml volumetric flask and made up to volume with tungstic acid reagent immediately after sampling. The mixture was allowed to stand for 1 hour then filtered through a Whatman No. 42 filter paper. An aliquot (0.5ml) filtrate was transferred to a glass stoppered test tube. To each tube 3ml reagent A, 5ml reagent B, then 5ml reagent C were added. The tubes were stoppered, inverted twice and incubated at 39°C for for 15 minutes. The absorbance at 589nm was read on a spectrophotometer. The ammonia nitrogen concentrations in the samples were determined by reference to a calibration graph, derived from standard solutions of ammonium sulphate containing 5.6 to 22.4mg ammonia-N/l.

2.4.14 Osmotic Pressure in Rumen Liquor

Osmotic pressure was determined using an Osmomat 303 osmometer (Gonotec, Germany) using sodium chloride as a reference (300mosmol).

2.4.15 Milk Total Solids

Total solids content of milk was determined gravimetrically according to British Standard

1741 (1961). A known weight of milk was dried, initially by evaporation over a boiling water bath for 30 minutes and then in an oven at 100°C for 3 hours.

2.4.16 Milk Fat

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

2.4.17 Milk Protein

Total nitrogen was determined by a macro-Kjeldahl method (Association of Official Agricultural Chemists, 1975). This value was multiplied by 6.38 to obtain the crude protein content.

2.4.18 Milk NPN

Milk NPN was determined by a micro-kjeldahl method according to White, White and Robertson (1956) using filtrate of tungstic acid precipitation of milk.

2.4.19 Milk Lactose

Milk lactose was determined by polarimetry (Grimbleby, 1956).

Reagents: Grimbleby's solution was prepared by dissolving 2.5g zinc acetate and 12.5g dodeca-tungstophosphoric acid in water, 20ml of glacial acetic acid was added and the mixture diluted to 200ml. The diluted mixture was filtered through Whatman No.42 filter paper.

Procedure: A 50ml wide-necked volumetric flask was weighed before and after 20ml of milk sample was added. Five ml Grimbleby solution was pipetted into the flask and the mixture was diluted to volume with water. The flask was stoppered, shaken vigorously and then left to stand at room temperature for 15 minutes and the mixture was filtered through Whatman No. 42 filter paper. An aliquot of the filtrate was taken for determination of the rotation using an automatic polarimeter (Thorn Automation Ltd, Nottingham). The polarimeter was standardized

with sucrose solution (2.6g Aristar sucrose per 100ml water) and this gave a rotation of 0.4069 at 20°C.

Calculation: A correction was made for the fat and protein by multiplying their percentages in the milk sample by standard factors (Giggs and Szigarto, 1963).

% Lactose = {(A x V) / (L x 61.9)} / (100 / wt of milk sample)

where A = observed rotation (degrees)

V = corrected volume of solution

L = length of tube with sample (dm)

2.4.20 Fatty Acid Composition of Milk Fat

Fat was extracted from milk by a method based on that of Bligh and Dyer (1959) and methyl esters were formed by a procedure modified from that of Christopherson 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 50ml test tube containing 3ml water. Ten ml methanol (Analar) and 5ml Chloroform (Analar) were added and mixed well. A further 5ml chloroform were added and mixed again. Five ml of 0.88% (w/v) KCl were added and, after mixing, the mixture was centrifuged at 1500g for 15 minutes. The lower layer was transferred, using a Pasteur pipette into a 50ml 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 3ml hexane and transferred to a 15ml conical test tube. The flask was washed with another 3ml hexane and the washings were added to the test tube. The hexane was evaporated from the sample at 50-60°C under nitrogen and 0.5ml hexane and 0.02ml of 2M sodium methoxide in dry methanol was added to methylate the lipid. The tube was stoppered and shaken at room temperature for 5 minutes. Hexane (0.5ml) 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/minute 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.

2.4.21 Plasma Ammonia

Determination of the ammonia concentration of blood plasma was measured by an enzymatic method using a commercial kit (Procedure No. 170-UV; Sigma Chemical, Poole, Dorset). The method was based on reductive amination of 2-oxoglutarate, using glutamate dehydrogenase and reduced nicotinamide adenine dinucleotide(NADH). The decrease in absorbance at 340nm, due to oxidation of NADH, is proportional to the plasma ammonia concentration.

Reagents

1) L-Glutamate dehydrogenase (L-GLDH) solution contained 1200 U/ml in 50% glycerol and phosphate buffer at pH 7.4.
2) Ammonia assay solution contained 2mM 2-oxoglutarate and 0.12mM NADH with buffer and stabilizer.

Procedure: Distilled water (0.2ml), standards and plasma were pipetted into a cuvette which containing 3ml of ammonia assay solution and mixed and then allowed to stand for 5 minutes for equilibration. The initial absorbance of each cuvette was read at 340nm using a spectrophotometer against water or potassium dichromate solution (3mg/75ml) as reference. Prior to the final reading 0.02ml L-GLDH solution was added, mixed by gentle inversion and allowed to stand for approximately 5 minutes.

Calculation

Plasma ammonia (μmol/l) = $(\Delta A_{TEST} - \Delta A_{BLANK}) \times 44 \times 59$

2.4.22 Plasma Urea

Determination of the urea concentration of blood plasma was carried out using an enzymatic procedure in a commercial kit (Cat. No.124788; Boehringer Corporation Ltd, Lewis, East Sussex). Urease cleaves urea in the presence of water with the formation of ammonium carbonate. The ammonium ion can then react with phenol and hypochlorite to give a blue coloured complex.

Reagents

1) Urease solution contained at least 10 U/ml in 50mM phosphate buffer.

2) Phenol reagent contained 0.106M phenol and 0.17mM sodium nitroprusside.

3) Hypochlorite reagent contained 11mM sodium hypochlorite and 0.125M sodium hydroxide.

Procedure: An aliquot of plasma (0.1ml) was diluted with 0.9ml of 0.9% (w/v) sodium chloride solution and 0.2ml of this diluted sample was added to 0.1ml solution containing at least 10 U/ml of urease in 5μl phosphate buffer at pH 6.5. One blank and one standard were also prepared containing 0.1ml of the urease solution and 0.2ml distilled water or 0.2ml of 0.5mM urea solution. The mixtures were mixed, stoppered and incubated for 10 minutes at 37°C prior to the addition of 5ml of each of the phenol and hypochlorite reagents. The mixtures were mixed and incubated for a further 15 minutes at 37°C. The absorbance was measured at 550nm with a spectrophotometer. After correction for the blank, the absorbance of the sample was divided by the absorbance of the standard. This value was multiplied by a conversion factor of 5 to give plasma urea concentration (mmol/l).

2.4.23 Plasma Glucose

The concentration of glucose in blood plasma was determined using an enzyme/colour reagent in a commercial kit (Cat. No.124036; Boehringer Corporation Ltd). The glucose was oxidized by glucose oxidase and, in the presence of peroxidase, the hydrogen peroxide formed

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

Reagents

1) Protein precipitating solution contained 1.6 g/l uranyl acetate and 9 g/l NaCl.
2) GOD-Perid reagent contained no less than 10 U/ml peroxidase and 1.0mg chromogen/ml in 100mM phosphate buffer, pH 7.0.

Procedure: Plasma (0.1ml) was added to 1ml uranyl acetate solution, mixed and centrifuged at 1500g for 15 minutes. Supernatant (0.2ml) was incubated in a test tube with 5ml GOD-Perid reagent at room temperature for 45 minutes. Blank determinations were performed on distilled water. The absorbance at 610nm was read on a spectrophotometer and glucose concentration determined by reference to a standard solution containing 9.1mg glucose/100ml.

2.4.24 Plasma Free Fatty Acids (FFA)

The concentration of free fatty acids (FFA) in blood plasma was determined enzymatically (Wako Pure Chemical Industries, Japan). In the presence of Coenzyme A, ATP and Acyl-CoA synthetase (ACS), FFA are converted to acyl CoA. This is oxidized to 2,3-trans-enoyl-CoA and hydrogen peroxide in a reaction catalysed by acyl-CoA oxidase (ACOD). In the presence of hydrogen peroxide and peroxidase the oxidative condensation of 3-methyl-N-ethyl-N-(6-hydroxyethyl)aniline (MEHA) with 4-amino antipyrine forms a red quinone complex. The optical density of the coloured complex was used to determine the FFA concentration.

Reagents

1) Solution A contained ACS, CoA, ATP, 4-amino antipyrine phosphate buffer solution pH 6.9, magnesium salt and Triton X-100.
2) Solution B contained ACOD, peroxidase, MEHA and Triton X-100.
3) FFA stock solution was 1mM oleic acid.

Procedure: One ml of solution A was added to 0.05ml of each standard and plasma for incubation. One blank and one sample blank were also prepared containing either 1ml

solution A and 0.05ml water or 1ml of solution A alone. The mixtures were incubated at 37°C for 10 minutes prior to the addition of 2ml of solution B to each tube. The mixtures were incubated for a further 10 minutes. At the end of the incubation period 0.05ml plasma was added to sample blank tube. The optical densities were measured spectrophotometically at 550nm and the FFA concentration in plasma was calculated by reference to the optical densities of a series of standard oleic acid solutions concentrations of 0.50-1.97mM.

2.4.25 Plasma β-Hydroxybutyrate

The concentration of plasma β-hydroxybutyrate was determined enzymatically using a commercial kit (Procedure No.310-UV, Sigma chemical). β-hydroxybutyrate dehydrogenase (β-HBDH) catalyses the oxidation of β-hydroxybutyrate (β-HBA) to acetoacetate. During this oxidation, an equimolar amount of NAD is reduced to NADH. NADH absorbs light at 340nm and, therefore, the increase in absorbance at 340nm is directly proportional to the β-hydroxybutyrate concentration in the sample.

Reagents

1) β-hydroxybutyrate reagent contained 4.6 mmol/l NAD, pH 7.6 buffer and oxamic acid.

2) β-hydroxybutyrate dehydrogenase reagent contained 50 U/ml β-HBDH and pH 7.6 buffer.

Procedure: Three ml of β-HBA reagent and 0.05ml of β-HBDH solution were pipetted into labeled tubes for reagent blank, calibrator, control and samples, and warmed to 37°C. 0.05ml of each of deionized water, calibrator containing 50mg/100ml D-β-Hydroxybutyrate, control and plasma were added to appropriately labelled tubes. Tubes were mixed by gentle inversion and incubated for 10 minutes at 37°C. Absorbance was read with a spectrophotometer using water as a reference. The concentration of FFA (mg/100ml) was calculated by subtracting the absorbance of the blank from the absorbance of samples, calibrator and control to obtain changes in absorbance due to β-hyroxybutyrate and then multiplied by a factor of 104.

2.4.26 Plasma Hormones

2.4.26.1 Insulin

The concentration of insulin was determined by double-antibody RIA by the method of Vernon et al. (1981) using iodinated rat insulin prepared by the technique described by Madon, Finley and Flint (1984). The iodination procedure used Enzymobeads impregnated with lactoperoxidase (Bio-Rad Laboratories, Califonia). To 25μl Enzymobeads was added 10μl of 0.5M phosphate buffer (pH 7.2), 5μg hormone, 500μCi^{125}Iodine and 15μl glucose solution (2% w/v). After 15 minutes the reaction was stopped by the addition of 200μl sodium azide (1 mg/ml) in phosphate-buffered saline (PBS) and the mixture was centrifuged at 1000 g for 2 minutes. The supernatant was transferred to a Sephacryl S-200/Sephadex G-10 column. The beads were then washed by the addition of 200μl KI (10 mg/ml) in PBS containing 1% bovine serum albumin (BSA) and re-centrifuged and the supernatant added to the column. The column was eluted with 0.5% BSA:PBS containing 0.1% sodium azide at a flow rate of 6 mm/hour and 0.5ml aliquots were collected. Sample (10μl) from the 6 aliquots was individually mixed with 25μl trichloroacetic acid (40% w/v) to precipitate labelled hormone. Free ^{125}Iodine was removed by decantation and the counts remaining were measured and from this the % iodination of the preparation was calculated.

^{125}I-labelled hormone was used under the following RIA conditions. Insulin standards ranging from 0-5ng insulin/ml were prepared by diluting a proprietary insulin stock solution with RIA buffer (0.5% BSA:PBS at pH 7.4). Each standard and sample was assayed in duplicate. The volume taken for analysis was 100μl. To this was added 100μl 'first antibody'. This was antiserum to bovine insulin-GP-3, which cross reacts with rat insulin, diluted 1:20,000 in RIA buffer. After addition of the antiserum the contents of the tubes were mixed thoroughly and incubated overnight at 4°C, together with duplicate 'blank' tubes containing 200μl RIA buffer. On the next day ^{125}I-insulin was diluted with RIA buffer so that 100μl of this mixture produced approximately 12,000cpm. Iodinated hormone was added to all tubes and also to an additional 2 'total counts' tubes. The tubes were incubated at 4°C overnight and then the second antibody, anti-guinea pig precipitating serum, was added. The 'second

antibody' mixture was prepared by dissolving 560mg EDTA in 19ml RIA buffer, adjusting the pH to 7.4 and adding 160μl normal guinea pig serum (1:10) and 240μl anti-guinea pig precipitaing serum. This was mixed thoroughly and 19ml of 25% PEG was added and mixed. With the exception of the total counts tubes, all tubes received 100μl 'second antibody' mixture and were incubated for 2 hours at room temperature. One ml of 4% (w/v) PEG was added to all but the 'total counts' tubes, after which the blanks, standards and samples were centrifuged at 2500 g for 30 minutes at 4°C. The supernatant was decanted and the activity of the pellets was counted. The concentration of insulin in the samples was calculated from the blank- and background-corrected activity by reference to that of the standards.

2.4.26.2 Growth Hormone

This hormone was assayed by double-antibody RIA. Iodinated growth hormone was prepared by the method described previously for insulin. Growth hormone standards ranging from 0-20 ng/ml were prepared by diluting a proprietary growth hormone preparation with RIA buffer (0.5% BSA:PBS, pH 7.4). The volume of standards and samples taken for analysis was 200μl. 'Blank' tubes contained 300μl RIA buffer. First antibody (100μl), antiserum to ovine growth hormone raised in rabbits, was added to all but the 'blank' tubes at a dilution of 1:20,000 in RIA buffer. All tubes were incubated overnight at room temperature. The following day 10,000cpm ^{125}I-growth hormone was added to each tube (in 100μl RIA buffer) and to 2 'total counts' tubes. The tubes were incubated overnight at 4°C. 'Second antibody' mixture was prepared by dissolving 140mg EDTA in 15ml RIA buffer, adjusting the pH to 7.4 and adding 5μl normal rabbit serum, 125μl anti-rabbit precipitating serum and 15ml of 16% PEG. With the exception of the 'total counts' tubes, each tube received 300μl 'second antibody' mixture. The contents of the tubes were mixed thoroughly and incubated at room temperature for 2 hours before being centrifuged at 2500 g for 30 minutes at 4°C. The supernatant was decanted and the activities in the 'total counts' tubes and the pellets in the remaining tubes were counted. The concentration of growth hormone in the sample was calculated by reference to the standard curve.

CHAPTER 3

CHAPTER 3 AMMONIA ABSORPTION FROM THE RUMEN AS A FACTOR AFFECTING VOLUNTARY FEED INTAKE OF COWS RECEIVING GRASS SILAGE DIETS

3.1 Introduction

The control of the voluntary intake of grass silage in dairy cows is not well understood. Although it is recognized that, besides digestibility, factors such as the concentration of various end-products of fermentation can influence intake, there is no clear understanding of their role (see Thomas and Chamberlain, 1989); indeed, silages of similar digestibility and fermentation profiles can differ widely in intake and milk production (Gordon, 1989c). Although, in regression analysis to correlate the concentration of fermentation end-products and voluntary intake of silage in dairy cows, the concentration of ammonia nitrogen consistently showed a significant negative effect on silage intake (Lewis, 1981; Gill *et al.*, 1988), the quantitative significance of this for the regulation of the voluntary intake of silage is as yet unclear.

The crude protein of grass silage is rapidly and extensively degraded to ammonia in the rumen (Thomas and Chamberlain, 1982a), and the digestion of diets containing a large proportion of silage is characterized by inefficient microbial utilization of silage nitrogen (Chamberlain *et al.*, 1986), resulting in absorption of substantial amounts of ammonia, equivalent to as much as 25% of the nitrogen ingested (Thomas and Thomas, 1985). Furthermore, it has been suggested that, in some practical feeding systems, such as grazing, the capacity of the bovine liver to metabolize ammonia may be exceeded (Symonds *et al.*, 1981). Ammonia escaping into the peripheral circulation can produce some perturbations of intermediary metabolism, particularly of glucose and of regulatory hormone concentrations, and depression of food intake (Kertz *et al.*, 1982; Fernandez *et al.*, 1988).

In view of these observations, it was decided to investigate the effects of increasing the amount of ammonia absorbed from the rumen on the food intake and milk production of dairy cows given grass silage diets. A series of four experiments was conducted, two with

non-lactating and two with lactating cows, in which progressively increasing amounts of urea were infused intraruminally. In the first two experiments, the effect of ammonia absorption from the rumen on silage intake (Experiment 1) and on silage intake and milk production (Experiment 2) was examined. Effects of rumen pH on ammonia absorption from the rumen and its effect on silage intake and milk production were examined in Experiment 3. Experiment 4 was concerned with a comparison of the effect of pattern of infusion of urea on silage intake: continuous infusion of urea was compared with equivalent doses given twice daily.

3.2 Experimental

3.2.1 Animals and their management and health

Eight Friesian cows were used in four experiments. Four non-pregnant, non-lactating cows were used in Experiments 1 and 4. The four cows used in Experiments 2 and 3 were in week 6-9 of lactation and in week 21-22 of lactation at the start of the respective experiments. Average body weights of the animals were approximately 540 (range 501-560), 562 (range 532-580), 606 (range 576-626) and 550kg (range 507-560) in Experiments 1, 2, 3 and 4 respectively. Each of the eight cows was fitted with a permanent cannula into the rumen. The animals were housed individually in metabolism stalls and the lactating cows were milked each day at 06.00 and 16.00h.

In Experiments 1, 2 and 3, some cows showed signs of restlessness at the highest doses of urea but there was no evidence of clinical ammonia toxicity.

3.2.2 Experimental Diets, Feeding and Intraruminal Infusion of Urea

In Experiments 1 and 4, cows received a diet of grass silage *ad libitum* as the sole component of the diet. In Experiments 2 and 3, grass silage was given *ad libitum* and was supplemented with 7 kg/d (fresh weight) of a barley-fishmeal cube. The silages were made from a sward of perennial ryegrass (*Lolium perenne*) cut at an early stage of growth with a disc mower. After wilting 2-4 hours, the grass was harvested with a precision-chop harvester set to

give a chop length of approximately 20mm and ensiled in a bunker silo. The silages used in Experiments 1 and 4 were ensiled with the addition of 2.3l of formic acid (Add-F, BP Chemicals; 850g formic acid/l)/t fresh grass, and the silages used in Experiments 2 and 3 were ensiled without additive. The concentrate consisted of 812g barley, 125g fishmeal and 63g salt and mineral mixture/kg DM.

The chemical composition of the dietary ingredients is shown in Table 3.1. The silages had a reasonable fermentation quality but in Experiments 1 and 4, silages had a low N content.

In all experiments, feed was given via an automatic feeder; in Experiments 1 and 4, eight equal meals a day were given at 06.00, 09.00, 12.00, 15.00, 18.00, 21.00, 24.00 and 03.00h and in Experiments 2 and 3, six equal meals a day were given at 06.00, 10.00, 14.00, 18.00, 22.00 and 02.00h. Feed intake was measured daily and the amount of silage offered was adjusted to ensure a refusal of approximately 15% of that offered.

All experiments involved intraruminal infusions of aqueous solutions of urea using a peristaltic pump (Watson Marlow, Falmouth, Cornwall); various amounts of urea were infused but the total volume of the infusate was kept constant at 4 l/d. The experimental design and treatments varied with each experiment.

3.2.3 Experimental Design and Procedure

3.2.3.1 Experiment 1

During a preliminary period of 14 days the animals were established on silage *ad libitum*. This was followed by a series of five 7-day experimental periods during which the animals received a continuous infusion supplying 0, 120, 240, 360 and 480g urea/d respectively. After this sequence of experimental treatments, the animals returned to a final period of 14 days during which no intraruminal infusions were given. Samples of rumen liquor and blood were taken at 09.00, 10.00, 11.00, 12.00, 13.00 and 14.00h on the last five days of the preliminary period to monitor the effect of feeding via automatic feeder on the changes of metabolites in the rumen fluid and plasma. During the last five days of each experimental period, samples of rumen liquor and blood were taken at 10.00h. Blood was obtained from an indwelling

Table 3.1 Chemical composition of the experimental diets

	Silage				Conc.
	Exp.1	**Exp.2**	**Exp.3**	**Exp.4**	
Dry matter^{+}, g/kg	232	222	231	277	858
Organic matter, g/kg DM	924	920	928	912	394
pH	3.78	3.76	3.59	3.85	nd
Total N, g/kg DM	13.4	22.4	23.3	17.6	34.2
True protein N, g/kg N	251	268	266	333	nd
NH_3-N, g/kg N	188	105	110	108	nd
Water-soluble carbohydrate g/kg DM	5	11	13	75	nd
Lactic acid, g/kg DM	98	133	145	40	nd
VFA, g/kg DM					
Acetic acid	44	12	20	12	nd
Propionic acid	3	-	1	-	nd
Butyric acid	1	1	tr	1	nd
Ethanol, g/kg DM	22	37	26	41	nd
NDF, g/kg DM	660	553	562	617	174
ADF, g/kg DM	427	334	354	377	44
Starch, g/kg DM	nd	nd	nd	nd	524
DOMD^{++}, g/kg DM	690	736	736	620	nd

$^{+}$, determined by toluene distillation; $^{++}$, measured in 4 sheep at maintenance level of feeding; nd, not determined; tr, trace

polythene catheter inserted into a jugular vein at the start of the sequence of experimental treatments.

3.2.3.2 Experiment 2

The experimental design was as in Experiment 1 except that the continuous intraruminal infusions supplied 0, 170, 330, 550 and 660g urea/d in the sequence of experimental treatments. Samples of blood and rumen liquor were taken at 11.00h on the last five days of each experimental period; blood was obtained by venepuncture from the tail. Milk yield was recorded daily and milk composition was determined on a bulked sample of the last four consecutive milkings in each period.

3.2.3.3 Experiment 3

Experiment 3 began 21 days after the completion of Experiment 2, using the same animals receiving the same diet as in Experiment 2. The experiment consisted of a series of continuous intraruminal infusions of urea in increasing doses but here, an attempt was made to maintain ruminal pH above 7 at all times by means of an indwelling rumen pH sensor (Figure 2.1) which controlled the infusion of sodium bicarbonate (100 g/l) into the rumen as described in Chapter 2. During the preliminary period, effects of sodium bicarbonate infusion on silage intake, milk production and composition of rumen fluid and plasma were evaluated. A 21-day preliminary period was followed by six 7-day experimental periods during which urea was infused continuously into the rumen at 0, 120, 240, 335, 470 and 600 g/d respectively; then animals were returned to a 14-day period during which no intraruminal infusion was given. Sampling times for rumen liquor, blood and milk were as in Experiment 2, blood being obtained by venepuncture from the tail.

3.2.3.4 Experiment 4

Experiment 4 was conducted using the same cows as in Experiment 1. A fourteen-day preliminary period was followed by a 14-day experimental period during which the animals

received a continuous intraruminal infusion of 240g urea/d. This was followed by a 7-day period in which the 240 g/d dose of urea was given in two equal portions during infusion periods extending from 09.00 to 11.00 and from 21.00 to 23.00h each day. The intention was to return the animals to a final 14-day period of continuous infusion but, owing to unforeseen circumstances, there was insufficient silage to complete the final period. Samples of jugular blood and rumen contents were taken on the last five days of the two experimental periods at 09.00, 10.00, 11.00, 12.00, 13.00 and 15.00h.

3.2.4 Chemical Analysis

Minced wet silage was analysed for toluene dry matter, total nitrogen, true protein, ammonia, lactic acid, ethanol, water soluble carbohydrate, VFA and pH. Dried samples of silage were analysed for ash, total nitrogen, neutral detergent fibre (NDF) and acid detergent fibre (ADF). The amount of digestible organic matter in the dry matter in silage (DOMD) was determined *in vivo*. Concentrate samples were analysed as appropriate for dry matter, total nitrogen, ash, NDF, ADF and starch. Rumen fluid was analysed for pH, ammonia, osmotic pressure and individual and total VFA. Blood plasma samples were analysed for ammonia, urea, glucose, non-esterified fatty acid (NEFA), β-hydroxybutyrate, insulin, growth hormone and amino acids. Milk samples were analysed for total solids, fat, crude protein, lactose and NPN.

3.2.5 Statistical Analysis

The results were subjected to analysis of variance and simple regression analysis to examine the treatment effects using Genstat 5 (Payne *at al.*, 1987). Owing to the omission of the final control period, the results of Experiment 4 were analysed by a paired t-test comparison of the two completed experimental periods.

3.3 Results

3.3.1 Experiment 1

Food Intake and the Composition of Rumen Fluid and Blood Plasma

The changes in composition of rumen contents and plasma during the day during the preliminary period are shown in Figure 3.1. Eight times daily feeding using an automatic feeder kept the concentrations of all the constituents measured, except rumen NH_3-N, reasonably constant. The concentrations of ruminal ammonia-N progressively decreased until 2h after feeding and remained constant thereafter (Figure 3.1b).

The effects of urea infusion on feed intake and on the composition of rumen fluid and plasma are shown in Table 3.2. The voluntary intake of silage was unaffected until the urea dose was increased to 480 g/d at which level silage intake was severely reduced ($P<0.001$) by around 50%. Rumen pH was significantly ($P<0.01$) increased only at the highest level of urea infusion. Ruminal concentrations of ammonia-N (Y, mg/l) increased with increasing doses of urea (X, g/d) in a linear fashion up to 360g urea/d; $Y = 0.682X + 39.6$ ($r = 0.987$, $n = 20$), but super-linearly thereafter. Plasma concentrations of urea (Y, mg/l) increased linearly at all dose rates ($Y = 0.852\ X + 93.9$; $r = 0.987$, $n = 20$), but the concentration of ammonia in plasma remained reasonably constant until the 360 g/d dose of urea when it increased markedly, reaching 3.5 mg/l with the highest level of urea infusion. Plasma concentrations of glucose tended to increase in response to urea infusion, the increases being significant ($P<0.05$ and $P<0.001$ respectively) for the two biggest doses of urea. At the highest level of urea infusion, plasma insulin was significantly reduced ($P<0.05$) whereas the concentration of plasma NEFA was increased.

The concentrations of rumen VFA are shown in Table 3.3. The higher levels of urea infusion increased ($P<0.05$) the molar proportion of propionic acid in the rumen but butyric acid proportion was increased with only the highest dose of urea. The molar proportions of valeric and isovaleric acids were progressively increased with increasing levels of urea up to 240 g/d, and decreased ($P<0.01$ and $P<0.001$ respectively) with a linear trend thereafter.

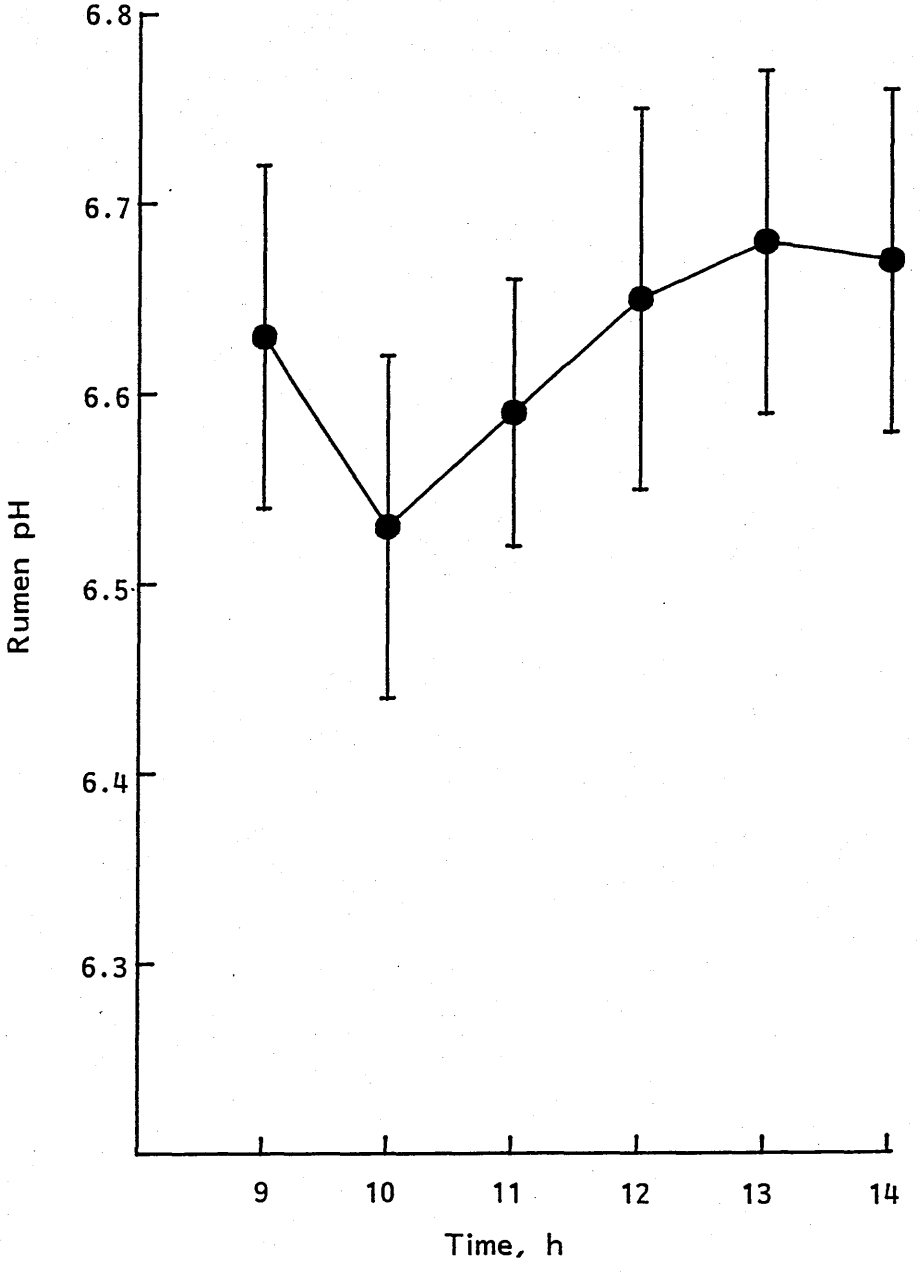


Figure 3.1a Changes during the day of rumen pH in cows fed silage 8 times daily using automatic feeder

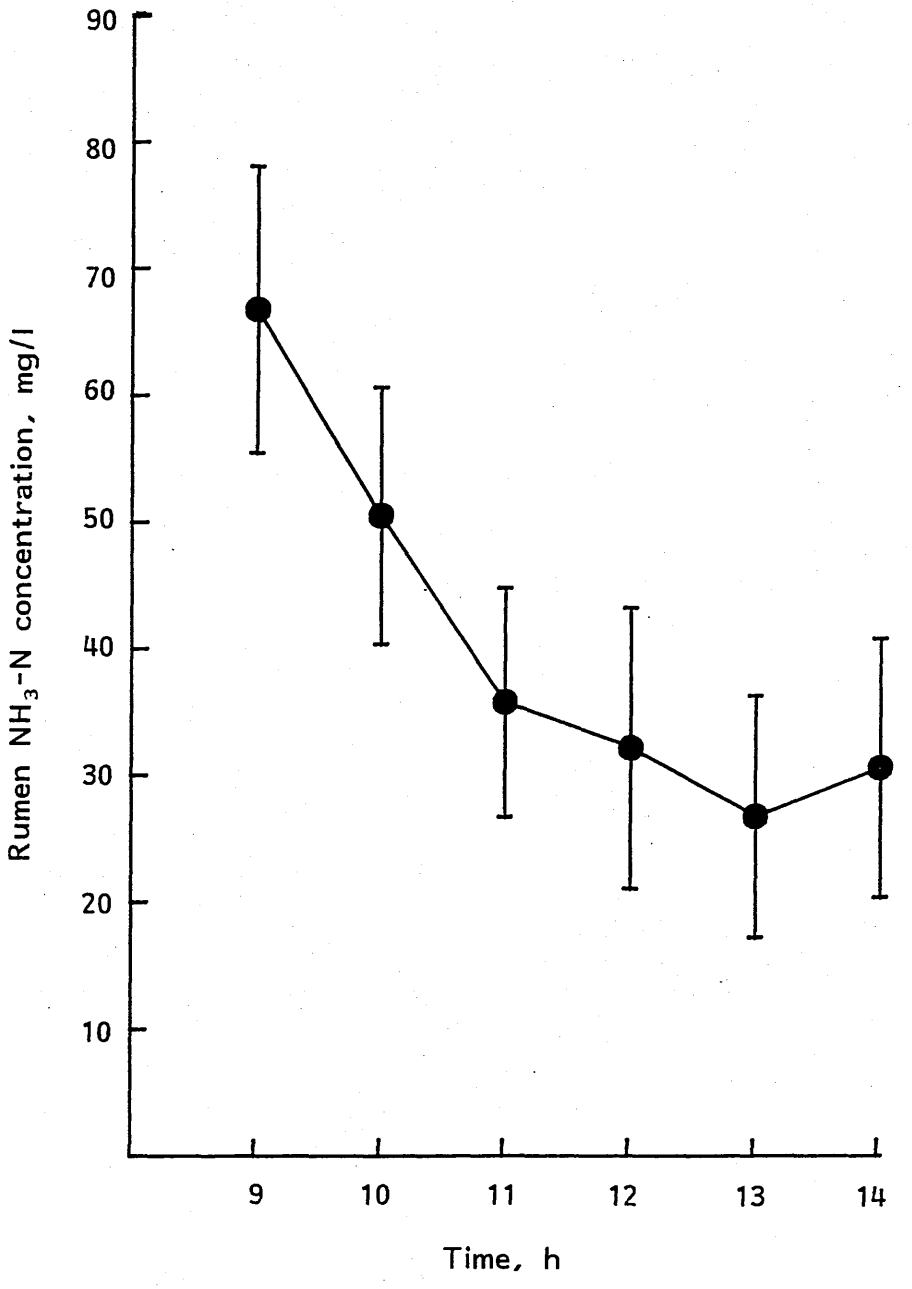


Figure 3.1b Changes during the day of the rumen concentration of ammonia (mg/l) in cows fed silage 8 times daily using automatic feeder

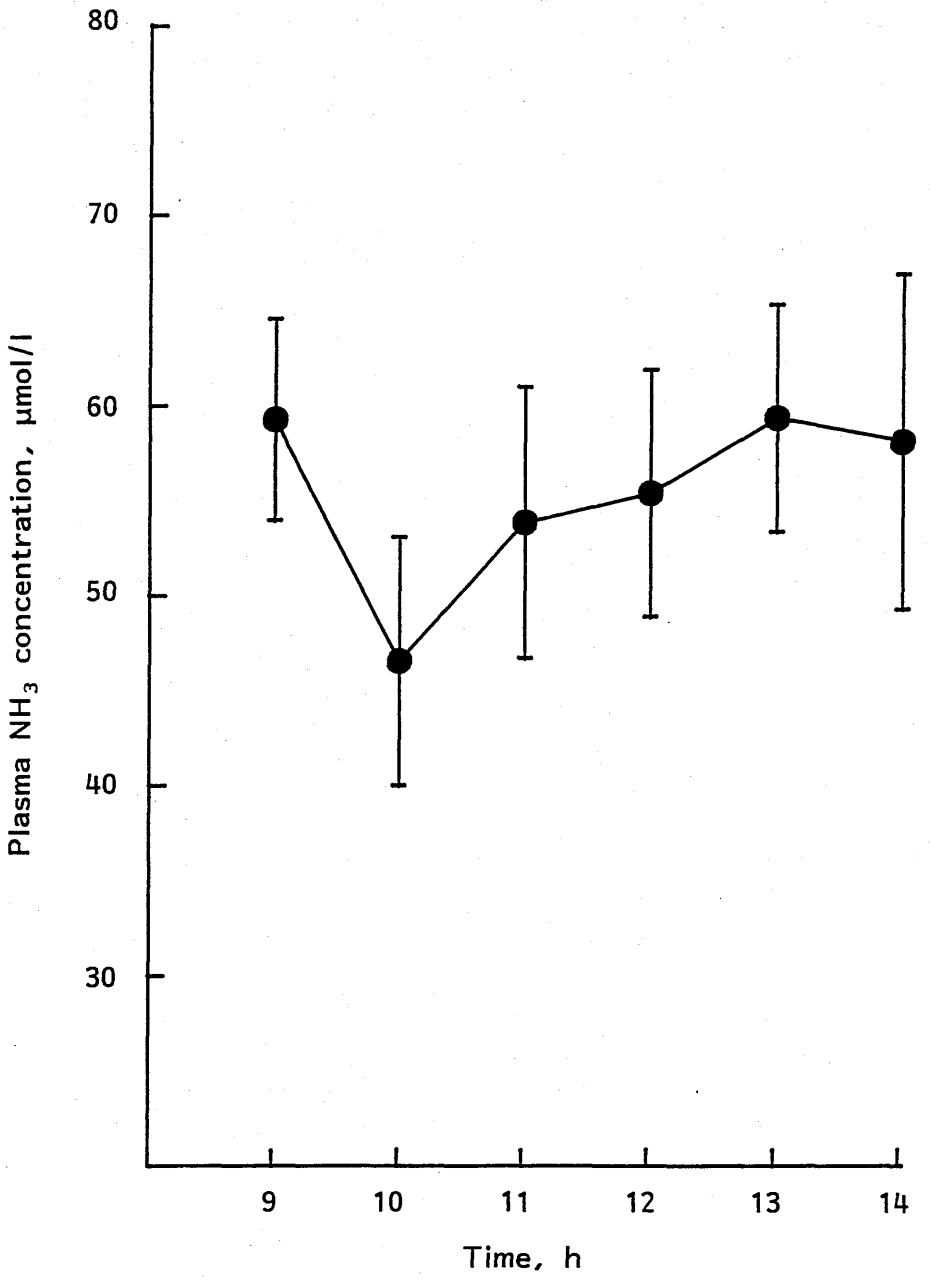


Figure 3.1c Changes during the day of the plasma concentration of ammonia (µmol/l) in cows fed silage 8 times daily using automatic feeder

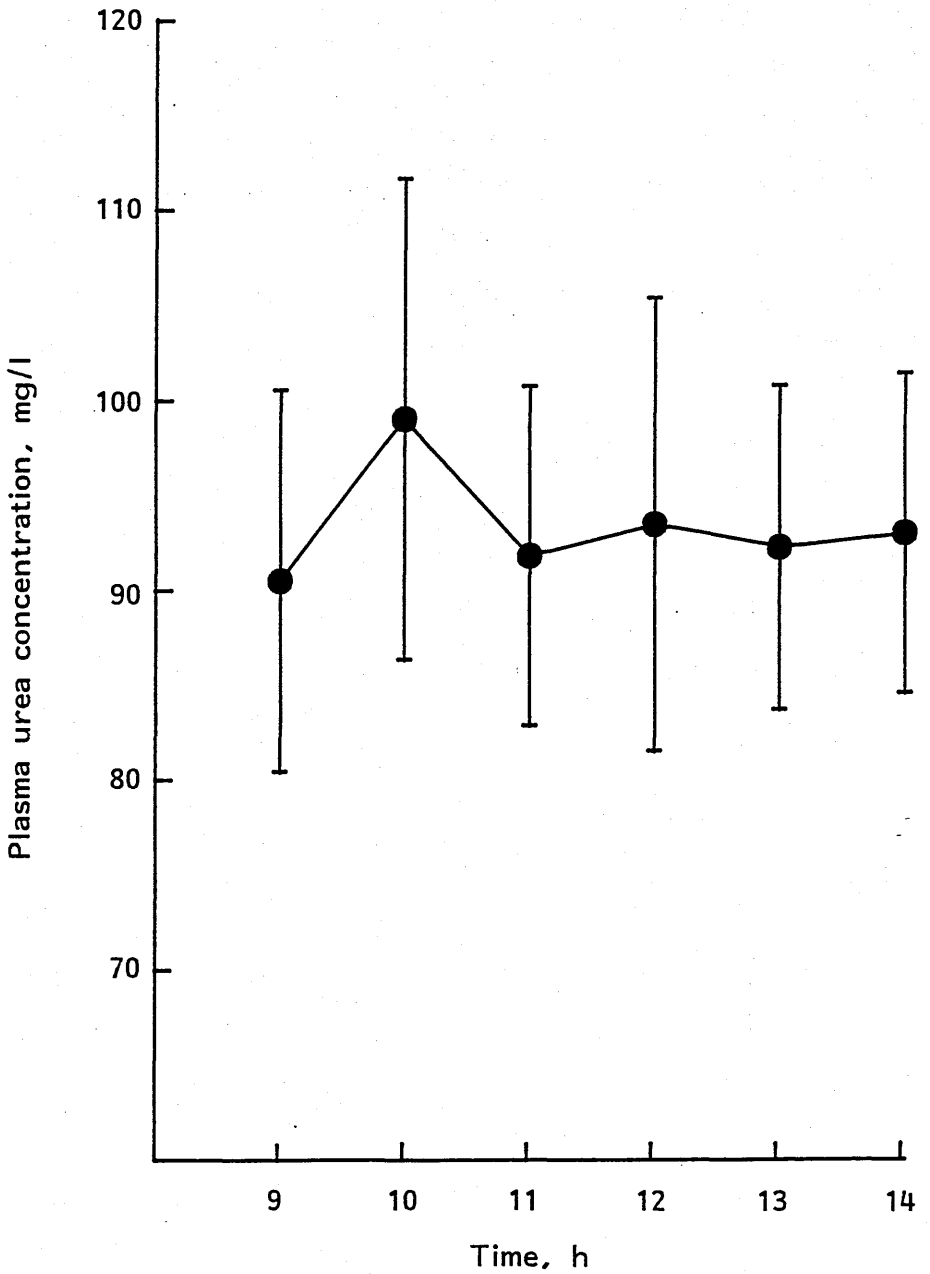


Figure 3.1d Changes during the day of the plasma concentration of urea (mg/l) in cows fed silage 8 times daily using automatic feeder

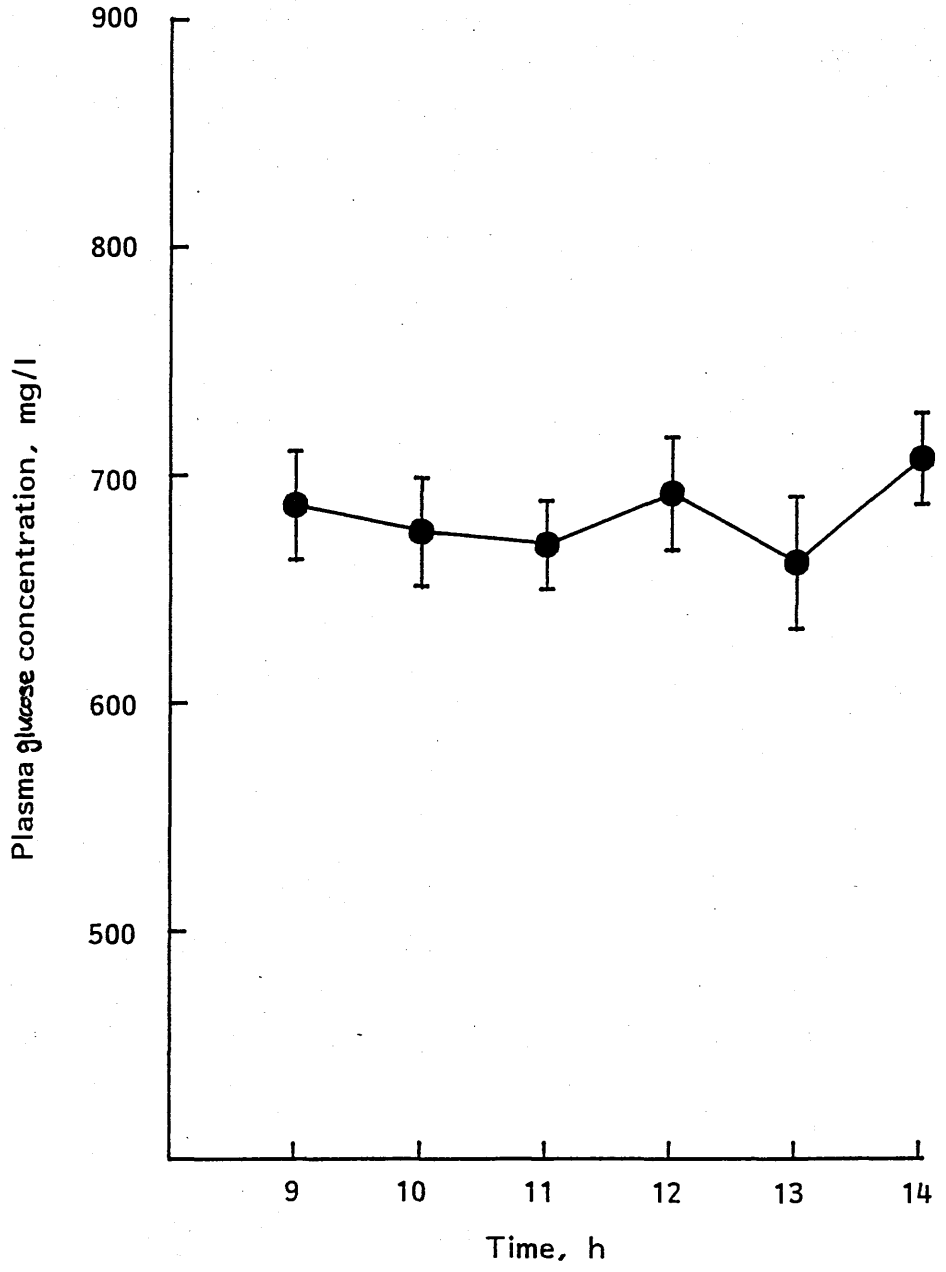


Figure 3.1e Changes during the day of the plasma concentration of glucose (mg/l) in cows fed silage 8 times daily using automatic feeder

Table 3.2 The effect of intraruminal infusions of urea on feed intake and on the composition of rumen fluid and plasma in Experiment 1

	Amount of urea infused, g/d						
	0	**120**	**240**	**360**	**480**	**s.e.d.**	
Silage intake, kg DM/d	8.0	8.1	8.4	9.0	4.4	0.8	**
Rumen pH	6.53	6.57	6.61	6.53	6.91	0.14	**
Rumen NH_3-N, mg/l	51	144	246	348	496	73.0	***
Plasma NH_3, mg/l	0.79	0.88	1.13	2.07	3.53	0.53	***
Plasma urea, mg/l	99	182	297	426	488	12.9	***
Plasma glucose, mg/l	616	660	654	708	910	41.3	***
β-Hydroxybutyrate, mg/l	71.5	112.9	113.1	106.0	105.9	30.6	
$NEFA^+$, mEq/l	0.06	0.09	0.07	0.08	0.21	0.06	
Insulin, ng/ml	0.52	0.51	0.45	0.41	0.38	0.07	*

$NEFA^+$, non-esterified fatty acid.

Table 3.3 The effect of intraruminal infusions of urea on the total concentration of VFA and the proportions of individual VFA in the rumen in Experiment 1

	Amount of urea infused, g/d						
	0	**120**	**240**	**360**	**480**	**s.e.d.**	
Individual VFA, molar %							
Acetic acid	69.0	70.5	68.2	68.0	69.3	0.90	*
Propionic acid	19.1	18.2	19.5	21.2	20.1	0.87	*
Isobutyric acid	0.5	0.6	0.5	0.1	0.5	0.17	
Butyric acid	6.4	5.6	6.2	6.0	7.1	0.21	*
Isovaleric acid	3.0	3.0	3.4	2.5	1.6	0.38	**
Valeric acid	2.0	2.1	2.3	2.2	1.3	0.18	***
Total VFA, mmol/100ml	11.0	9.7	10.0	10.2	10.2	0.87	

The effects of urea infusion on plasma free amino acid concentrations are presented in Table 3.4. The concentrations of most essential and non-essential amino acids were significantly (at least $P<0.05$) depressed in a linear trend up to 360g urea infusion but raised at the highest dose. The depression in the concentrations of Thr, Val, Phe, Ile, Leu and Ala were especially large ($P<0.001$). Concentrations of Arg and Tau were increased ($P<0.05$ and $P<0.001$ respectively) linearly with increasing dose of urea whereas α-Aba and Orn concentrations were decreased ($P<0.01$). Intraruminal infusions of urea did not affect the concentrations of plasma His and Ser.

3.3.2 Experiment 2

Food Intake and the Composition of Rumen Fluid and Blood Plasma

Changes in feed intake and in the composition of rumen fluid and plasma in response to intraruminal infusion of urea are shown in Table 3.5. Similarly to the pattern with non-lactating cows in Experiment 1, ruminal concentrations of ammonia-N and plasma concentrations of urea increased linearly with urea dosing (X, g/d): Y (rumen NH_3-N, mg/l) = 0.417 X + 127.0 ($r = 0.874$, $n= 20$); Y (plasma urea, mg/l) = 0.586 X +270.0 ($r = 0.971$, $n= 20$). Plasma concentration of ammonia remained constant until the highest level of urea infusion when it increased by about 100% to 3.4 mg/l and this was accompanied by a reduction ($P<0.05$) in voluntary intake of silage; small amounts of concentrate were refused also when the highest level of urea was given. Plasma concentration of glucose tended to increase progressively with urea dosage.

Rumen VFA pattern in Experiment 2 (Table 3.6) differed from that in Experiment 1 (Table 3.3). The higher total VFA concentrations and higher proportions of propionic acid and butyric acid in Experiment 2 could be due to concentrate feeding. Urea infusion decreased the molar proportion of butyric acid and increased total VFA concentration. The effect of intraruminal infusions of urea on concentrations of plasma free amino acids are shown in Table 3.7. In contrast to the results observed in Experiment 1, the concentrations of all essential and most non-essential amino acids and intermediate metabolites measured did not

Table 3.4 The effect of intraruminal infusions of urea on the concentrations of plasma free amino acids (μmol/l) in Experiment 1[+]

	Amount of urea infused, g/d						
	0	**120**	**240**	**360**	**480**	**s.e.d.**	
Essential AA							
His	56	49	49	62	59	6.2	
Thr	105	86	76	72	90	5.6	***
Arg	106	108	115	120	121	4.7	*
Trp	41	34	36	34	37	2.5	
Met	12	11	10	12	13	0.9	*
Val	226	199	181	161	202	9.1	***
Phe	67	58	57	51	59	1.8	***
Ile	107	99	94	77	85	4.4	***
Leu	111	97	89	75	106	5.1	***
Lys	111	96	96	88	97	6.1	*
TEAA	943	838	801	750	869	26.4	***
Non-essential AA							
Asp	18	15	17	12	14	1.3	**
Glu	126	110	143	104	140	10.5	*
Ser	76	67	65	61	70	5.1	
Gly	197	150	113	121	143	17.8	**
Ala	156	127	125	113	139	6.5	***
Tyr	58	49	51	47	46	3.2	*
TNEAA	631	518	513	458	553	30.0	**
TAA	1573	1355	1314	1208	1422	45.7	***
Intermediate metabolites							
Asn	15	14	9	15	11	1.7	*
Gln	250	214	147	337	251	20.9	***
Tau	30	33	35	40	41	1.6	***
α-Aba	11	9	9	6	6	1.0	**
Orn	107	92	90	92	78	5.9	**

[+], see Figure 2.4 in Chapter 2 for abbreviations; TEAA, total essential amino acid; TNEAA, total non-essential amino acid; TAA, total amino acid

Table 3.5 The effect of intraruminal infusions of urea on feed intake and on the composition of rumen fluid and plasma in Experiment 2

	Amount of urea infused, g/d						
	0	**170**	**330**	**500**	**660**	**s.e.d.**	
Silage intake, kg DM/d	9.0	10.6	10.1	9.0	7.5	0.70	**
Rumen pH	6.02	5.74	5.81	6.14	5.92	0.09	**
Rumen NH_3-N, mg/l	159	244	322	376	511	52.1	***
Plasma NH_3, mg/l	1.65	1.60	1.46	1.63	3.37	0.83	
Plasma urea, mg/l	251	390	473	564	647	24.0	***
Plasma glucose, mg/l	586	580	623	678	778	71.0	
NEFA, mEq/l	0.14	0.13	0.08	0.09	0.12	0.02	*
β-hydroxybutyrate, mg/l	285	263	219	203	168	38.5	

Table 3.6 The effect of intraruminal infusions of urea on the total concentrations of VFA and the proportions of individual VFA in the rumen in Experiment 2

	Amount of urea infused, g/d						
	0	**170**	**330**	**500**	**660**	**s.e.d.**	
Individual VFA, molar %							
Acetic acid	59.6	62.9	59.5	61.4	62.9	1.20	*
Propionic acid	22.8	22.4	25.4	23.6	22.6	1.02	
Isobutyric acid	0.5	0.9	0.5	0.4	0.6	0.24	
Butyric acid	13.9	10.3	11.1	10.6	10.4	0.92	**
Isovaleric acid	1.5	2.0	1.7	2.3	2.1	0.28	
Valeric acid	1.7	1.5	1.8	1.6	1.5	0.11	
Total VFA, mmol/100ml	12.9	14.7	16.4	15.6	17.0	0.85	**

Table 3.7 The effect of intraruminal infusions of urea on the concentrations of plasma free amino acids (μmol/l) in Experiment 2

	Amount of urea infused, g/d						
	0	**170**	**330**	**500**	**660**	**s.e.d.**	
Essential AA							
His	53	55	59	59	53	6.6	
Thr	130	137	138	139	96	15.1	
Arg	90	98	105	110	75	9.8	*
Trp	43	46	48	50	40	4.5	
Met	19	20	20	23	15	2.8	
Val	231	233	247	253	157	28.0	*
Phe	55	59	57	57	52	3.6	
Ile	124	117	112	114	72	13.8	*
Leu	101	103	115	120	90	13.8	
Lys	88	81	91	101	76	11.3	
TEAA	935	947	991	1026	725	98.7	
Non-essential AA							
Asp	19	21	19	19	13	1.1	***
Glu	81	84	77	78	71	3.7	*
Ser	89	90	78	77	75	9.5	
Gly	333	289	277	268	201	24.7	**
Ala	202	204	197	193	159	13.8	*
Tyr	46	49	50	50	36	4.2	*
TNEAA	770	736	698	685	554	32.0	***
TAA	1705	1683	1689	1711	1279	117.4	*
Intermediate metabolites							
Asn	39	41	39	40	22	3.9	**
Gln	384	404	377	405	326	21.0	*
Tau	37	42	34	35	27	7.8	
α-Aba	20	20	14	13	9	1.5	***
Orn	48	47	55	64	56	6.0	

change or tended to increase up to the 500g urea infusion level. However, the highest level of urea addition decreased the concentrations of most plasma amino acids. Among the essential amino acids, concentrations of Thr, Met, Val and Ile showed greater depressions than other acids with the respective values of 26, 25, 32 and 42% compared with control. The concentrations of Gly and α-Aba decreased with increasing dose of urea infusion.

Milk Production

The milk production data are given in Table 3.8. Milk yield was reduced ($P<0.05$) at the highest level of urea infusion and a similar pattern was seen in the yield of lactose. The concentration of fat was increased ($P<0.05$) with the biggest dose of urea such that the yield of fat was not significantly reduced ($P>0.05$) despite the reduction of milk yield. The yield of crude protein tended to increase with the intermediate levels of urea before decreasing with the highest level. Although the concentration of NPN increased progressively ($P<0.01$) with urea dose, the apparent increases in the yield of milk protein were not wholly explained by the increases in NPN output: yield of protein corrected for NPN were 663, 710, 732, 691 and 585 g/d in order of increasing dosage of urea. Corresponding figures for the concentration of protein corrected for NPN were 29, 31, 32, 32 and 32 g/kg respectively.

3.3.3 Experiment 3

Food Intake and the Composition of Rumen Fluid and Blood Plasma

The effect of intraruminal infusion of sodium bicarbonate on feed intake, milk yield and compositions of rumen fluid and plasma during the preliminary period are shown in Table 3.9. Intraruminal addition of sodium bicarbonate did not affect the silage intake and milk production but rumen pH, osmotic pressure in the rumen fluid and the concentration of plasma urea were significantly ($P<0.01$, $P<0.01$ and $P<0.05$ respectively) increased by sodium bicarbonate infusion.

Changes in silage intake and the composition of rumen fluid and plasma are presented in Table 3.10. The attempt to maintain rumen pH above 7 was not entirely successful possibly

Table 3.8 The effect of intraruminal infusions of urea on milk production in Experiment 2

	Amount of urea infused, g/d						
	0	**170**	**330**	**500**	**660**	**s.e.d.**	
Milk yield, kg/d	23.1	23.3	23.0	21.5	18.3	1.68	
Total solids, g/kg	123	127	126	132	134	3.5	*
g/d	2822	2942	2882	2851	2444	219.9	
Fat, g/kg	37.4	39.9	37.5	42.8	46.9	2.99	*
g/d	863	935	858	937	849	88.8	
Protein^{+}, g/kg	31.0	32.7	34.1	35.3	34.8	0.7	***
g/d	709	756	778	756	640	60.6	
Lactose, g/kg	47.2	47.7	47.1	47.8	44.8	0.7	**
g/d	1082	1099	1080	1028	818	81.2	*
NPN, g/kg	0.24	0.32	0.34	0.44	0.45	0.02	***
g/d	5.73	7.45	7.86	9.53	8.18	0.91	*

$^{+}$, N X 6.38

Table 3.9 The effect of intraruminal infusion of sodium bicarbonate on feed intake, milk yield and composition of rumen fluid and plasma during the preliminary period in Experiment 3

	Control	SBC^+	s.e.d.	
Silage intake, kg DM/d	9.1	9.3	0.23	
Milk yield, kg/d	18.5	18.4	0.45	
Rumen pH	6.10	6.37	0.1	**
Rumen NH_3-N, mg/l	116	139	11.5	
Osmotic pressure, mosmol/l	310	325	7.3	**
Plasma NH_3, mg/l	1.4	1.4	0.18	
Plasma urea, mg/l	235	262	9.3	*
Plasma glucose, mg/l	670	685	12.8	

SBC^+, sodium bicarbonate.

Table 3.10 The effect of intraruminal infusions of urea on the composition of rumen fluid and plasma in Experiment 3

	Amount of urea infused, g/d							
	0	**120**	**240**	**355**	**470**	**600**	**s.e.d.**	
Silage intake, kg DM/d	9.1	9.1	9.1	9.2	8.7	7.8	0.39	*
Water consumption, l/d	33.0	25.8	32.9	29.1	33.0	30.6	1.68	**
Rumen pH	6.44	6.50	6.20	6.08	6.27	6.62	0.19	
Rumen NH_3-N, mg/l	203	216	239	284	374	668	160	
Osmotic pressure, mosmol/l	341	338	329	327	336	392	31.32	
Plasma NH_3, mg/l	1.77	1.05	1.12	1.55	1.87	2.67	0.65	
Plasma urea, mg/l	253	311	339	467	530	619	25.6	***
Plasma glucose, mg/l	692	653	662	698	668	744	26.9	*
Insulin, mg/ml	2.6	1.5	1.3	1.4	1.3	1.0	0.30	***
Growth hormone, ng/ml	3.7	3.8	3.5	3.4	3.5	3.6	0.40	

because the pH sensor did not register a pH reading that was representative of the whole rumen contents. Nevertheless, in general, rumen pH was maintained at substantially higher values than in Experiment 2 (Table 3.5). Voluntary intake of silage was similar to that observed in Experiment 2 but there was no evidence of any stimulation of feed intake by urea infusion. Similarly to the pattern in Experiments 1 and 2, the relations between the concentration of rumen ammonia-N and the amount of urea infused (X, g/d) and between the plasma concentrations of urea and the amount of urea infused were linear: Y (rumen NH_3-N, mg/l) = 0.347 X + 125.0 (r = 0.933, n = 22); Y (plasma urea, mg/l) = 0.622 X + 243.0 (r = 0.938, n = 22). Plasma concentrations of ammonia increased only at the highest level of urea infusion and again, this was accompanied by a reduction ($P<0.05$) in silage intake and small refusals of concentrate. Plasma concentrations of glucose increased ($P<0.05$) with the biggest dose of urea. Insulin concentration in plasma was reduced ($P<0.001$) by all levels of urea infusion compared with the zero level.

The concentrations of plasma amino acids are given in Table 3.11. Although there were fluctuations in the concentrations of amino acids at the first three levels of urea infusion, the pattern of changes in amino acid profile, and the dose of urea at which the depressions occurred were similar to those in Experiment 2 (Table 3.7). Concentrations of most amino acids were markedly reduced by the two highest levels of urea. The depressions in Val, Phe, Ile and Leu were especially marked, and reductions of these acids relative to control were 17, 18, 18 and 24% respectively. The concentration of α-Aba was progressively decreased with increasing dose of urea.

Milk production

Despite similar feed intakes to those in Experiment 2, milk yields were lower in this experiment (Table 3.12), presumably owing to the later stage of lactation. Changes in milk production showed a similar pattern to those in Experiment 2. Yields of milk and lactose were reduced ($P<0.05$) by the biggest dose of urea but the concentration of fat was increased such that the yield of fat was maintained. Again, the concentration of NPN in the milk increased

Table 3.11 The effect of intraruminal infusions of urea on the concentrations of plasma free amino acids (μmol/l) in Experiment 3

	Amount of urea infused, g/d							
	0	**120**	**240**	**355**	**470**	**600**	**s.e.d.**	
Essential AA								
His	54	45	48	53	47	50	2.7	*
Thr	94	89	82	91	80	83	6.5	
Arg	79	77	76	84	71	77	4.3	
Trp	47	45	38	47	38	43	3.9	
Met	14	14	13	17	15	16	1.5	
Val	199	186	191	190	165	173	13.4	*
Phe	48	46	41	42	40	39	2.8	*
Ile	93	87	90	87	76	76	5.0	*
Leu	112	102	100	99	85	94	8.5	
Lys	97	85	85	91	81	84	4.8	
TEAA	836	777	764	810	698	735	35.7	*
Non-essential AA								
Asp	15	17	18	17	14	14	1.5	
Glu	83	74	86	84	83	77	3.7	*
Ser	66	59	52	59	51	49	4.6	*
Gly	222	208	175	179	169	183	21.3	
Ala	179	162	157	177	143	147	10.0	*
Tyr	43	41	38	42	35	34	2.7	*
TNEAA	608	560	529	556	496	504	30.4	*
TAA	1443	1337	1293	1367	1194	1238	53.6	**
Intermediate metabolites								
Asn	23	19	17	23	20	21	4.4	
Gln	212	195	211	251	234	255	27.0	*
Tau	33	29	28	31	31	35	2.1	*
α-Aba	11	8	8	7	6	5	0.7	***
Orn	57	49	54	56	59	53	2.9	*

Table 3.12 The effect of intraruminal infusions of urea on milk production in Experiment 3

	Amount of urea infused, g/d							
	0	**120**	**240**	**355**	**470**	**600**	**s.e.d.**	
Milk yield, kg/d	18.4	18.4	18.5	18.3	17.5	15.3	0.96	*
Total solids, g/kg	132	129	131	126	129	142	10.4	
g/d	2417	2361	2418	2277	2225	2164	95.0	
Fat , g/kg	44.1	41.8	43.7	39.3	40.5	54.6	9.80	
g/d	811	757	810	709	697	827	121.0	
Protein, g/kg	34.2	34.4	34.5	34.4	35.5	37.9	1.80	
g/d	623	628	630	622	614	575	21.2	
Lactose, g/kg	46.9	46.4	46.0	45.5	45.8	43.5	1.40	
g/d	861	850	848	829	797	664	60.4	*
NPN, g/kg	0.25	0.26	0.29	0.30	0.36	0.45	0.03	***
g/d	4.61	4.89	5.34	5.42	6.44	6.80	0.30	***

progressively ($P<0.001$) with urea dosage but, after correcting for NPN, there was a tendency for the concentration of milk protein to increase at the highest levels of addition of urea, the concentrations being 32, 32, 33, 32, 34 and 35 g/kg in order of increasing urea input.

3.3.4 Experiment 4

Food Intake and the Composition of Rumen Fluid and Blood Plasma

Voluntary intake of silage was significantly ($P<0.01$) reduced by the twice-daily infusion of urea as compared with continuous infusion (Table 3.13).

The changes in composition of rumen contents and plasma during the day are shown in Figure 3.2. In general, the concentrations of all the constituents measured were reasonably constant during the continuous infusion treatment. In contrast, when the urea was administered twice daily, there were marked peaks in ruminal pH (Figure 3.2a) and ammonia-N concentrations in rumen fluid (Figure 3.2b) and plasma (Figure 3.2c), both of which showed clearly defined peaks 2h after the start of the infusion of urea. However plasma urea concentration when urea was infused twice a day was higher than with the continuous infusion treatment only at 2h after the start of infusion and the differences between the two treatments were small (Figure 3.2d). Over the 6h during which the concentrations were monitored, plasma concentrations of glucose were higher and showed much greater fluctuation with the twice-daily dosing treatment (Figure 3.2e). In contrast to the concentrations of glucose, insulin concentrations were depressed by rapid infusion (Figure 3.2f).

The mean concentrations of plasma free amino acids during the day are shown in Table 3.14. Twice a day infusion of urea markedly reduced (at least $P<0.05$) the concentrations of most plasma amino acids compared with the continuous infusion. Concentrations of Gly, Asp, Glu, Gln and Tau were increased by twice-daily infusion of urea but His, Met, Ser and Ala concentrations were not affected.

Table 3.13 The effect of two different methods of intraruminal infusion of urea on feed intake in Experiment 4

	Continuous infusion	Twice daily infusion	s.e.d.	
Silage intake, kg DM/d	9.1	8.1	0.12	**

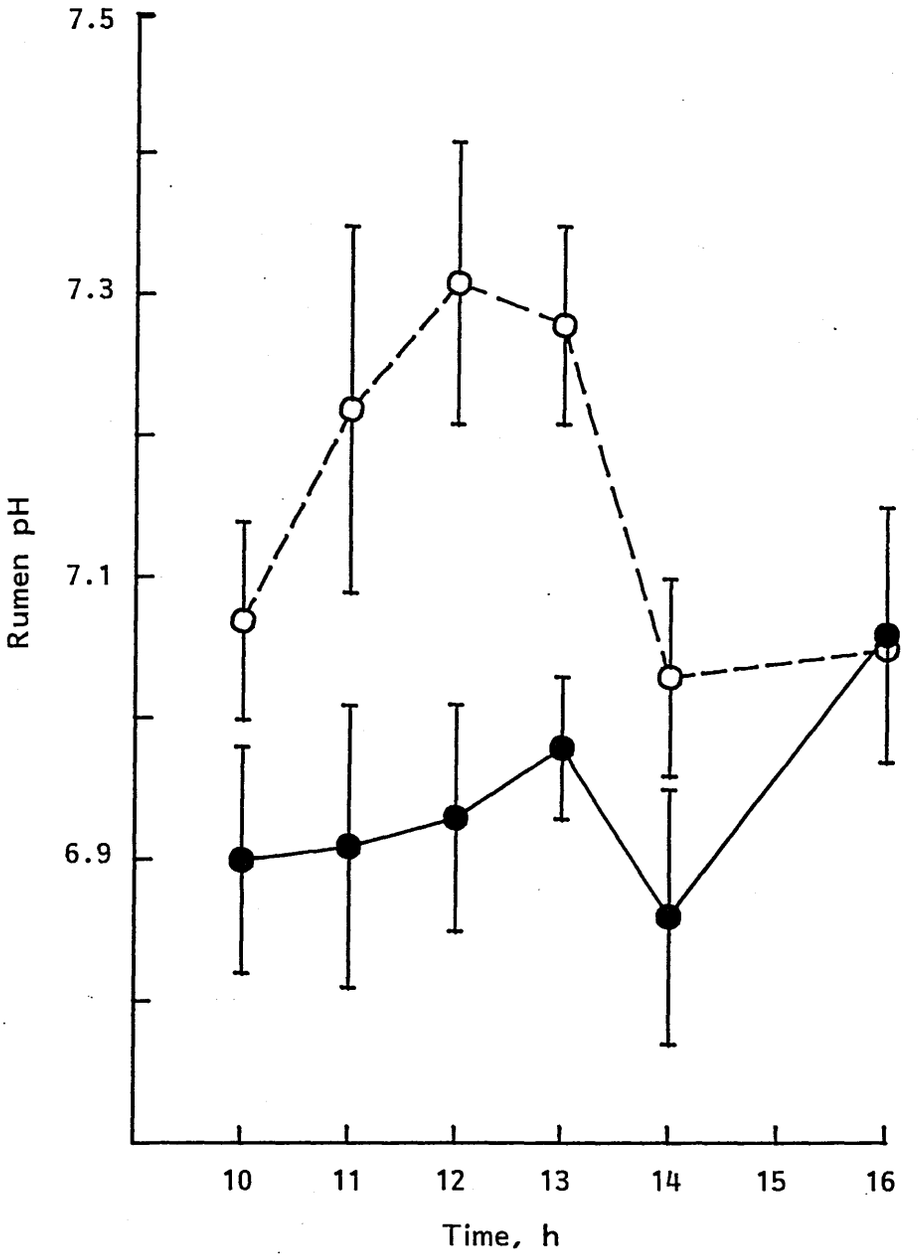


Figure 3.2a Changes during the day of rumen pH in cows given 240g urea/d either as a continuous intraruminal infusion (●) or as 2 doses/d (○) at 10.00 and 22.00h

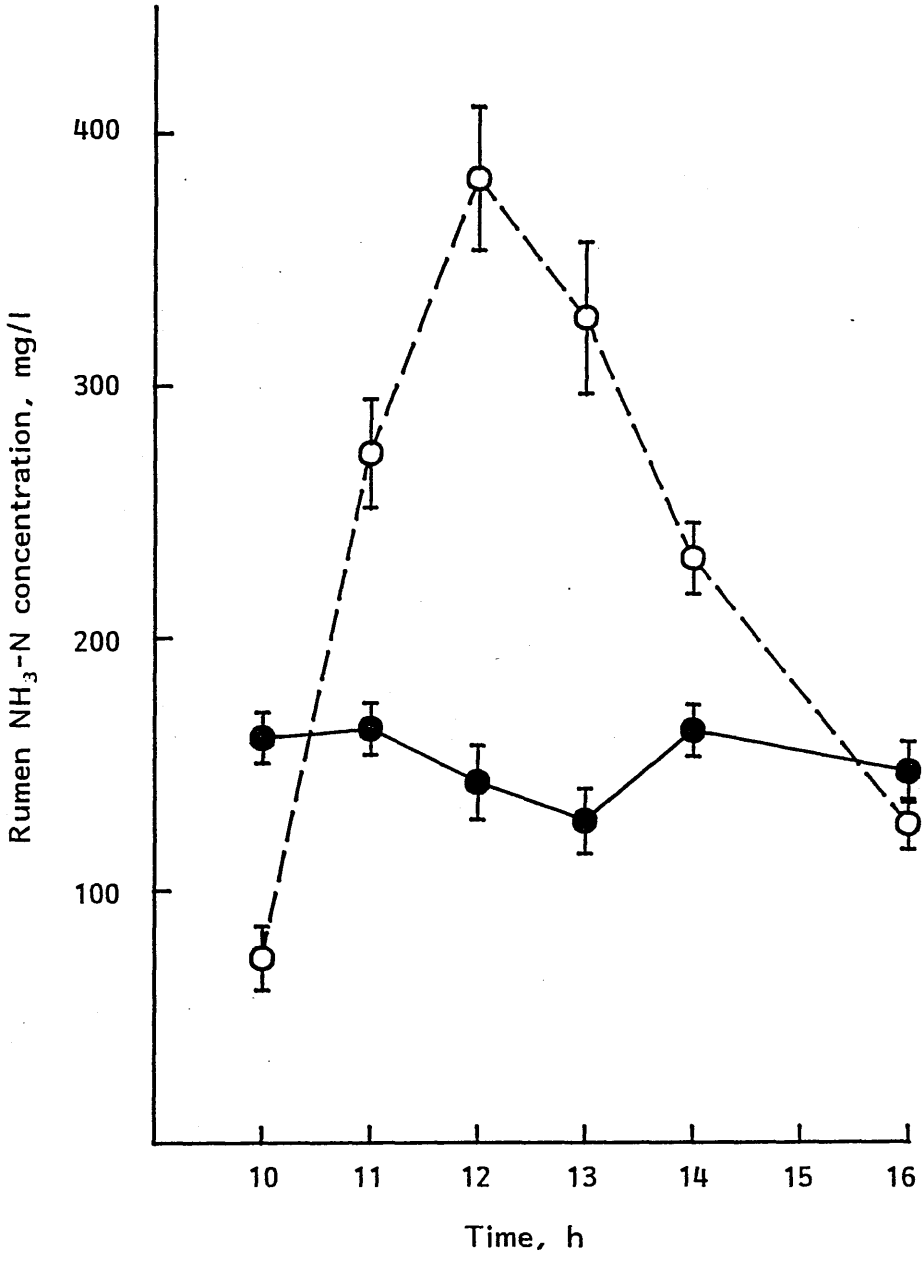


Figure 3.2b Changes during the day of rumen concentration of ammonia (mg/l) in cows given 240g urea/d either as a continuous intraruminal infusion (●) or as 2 doses/d (○) at 10.00 and 22.00h

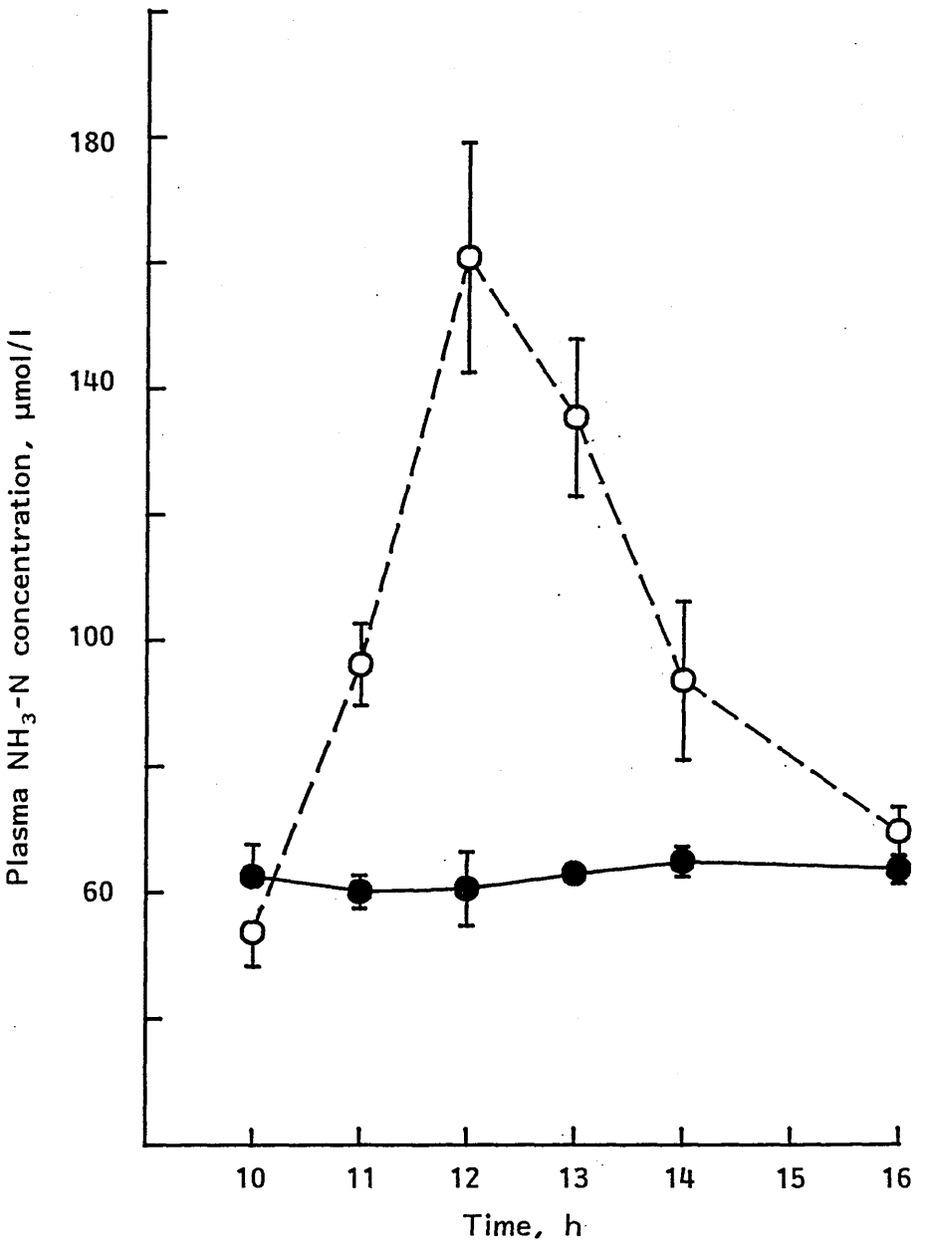


Figure 3.2c Changes during the day of plasma concentration of ammonia (µmol/l) in cows given 240g urea/d either as a continuous intraruminal infusion (●) or as 2 doses/d (○) at 10.00 and 22.00h

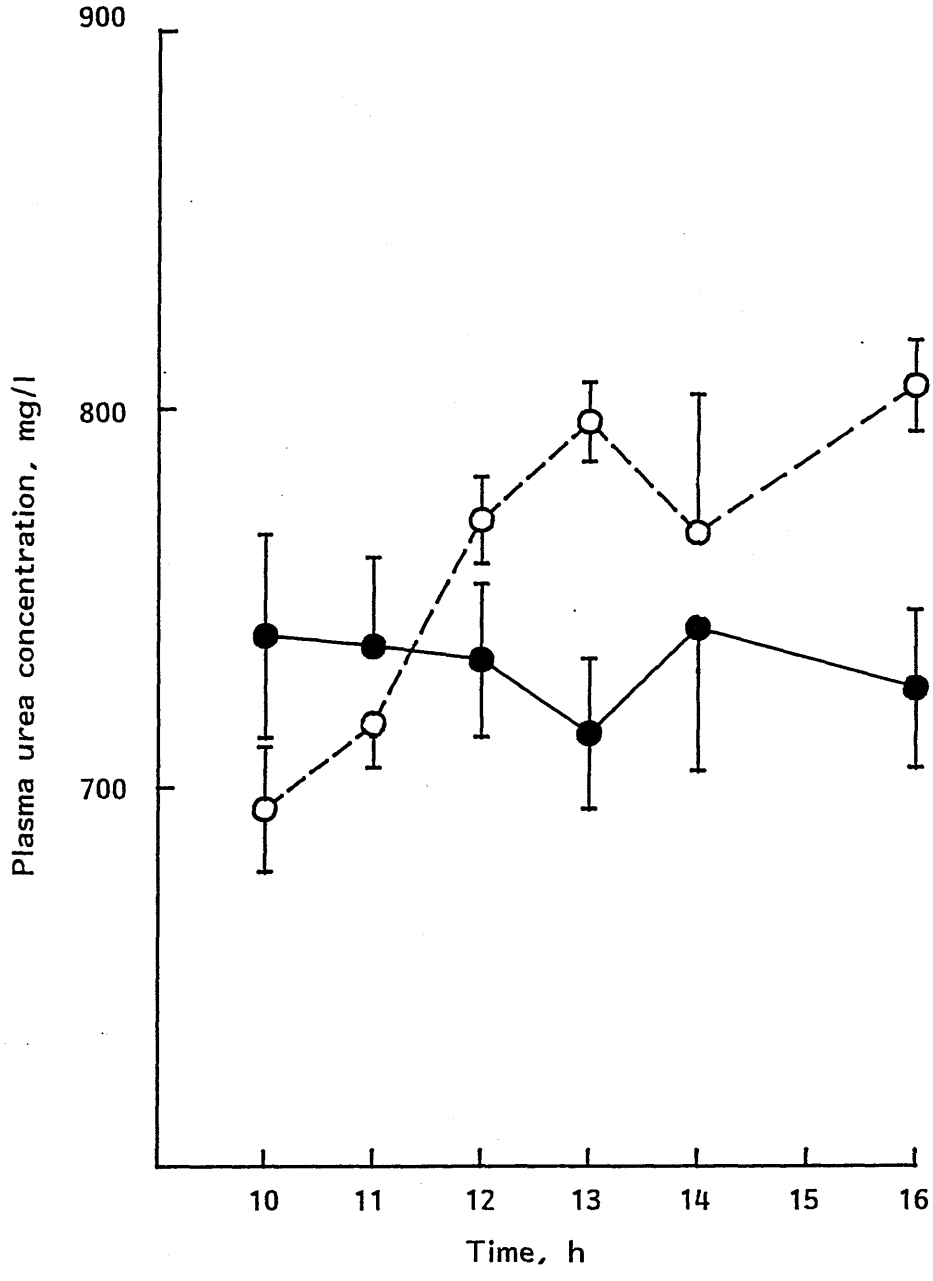


Figure 3.2d Changes during the day of plasma concentration of urea (mg/l) in cows given 240g urea/d either as a continuous intraruminal infusion (●) or as 2 doses/d (○) at 10.00 and 22.00h

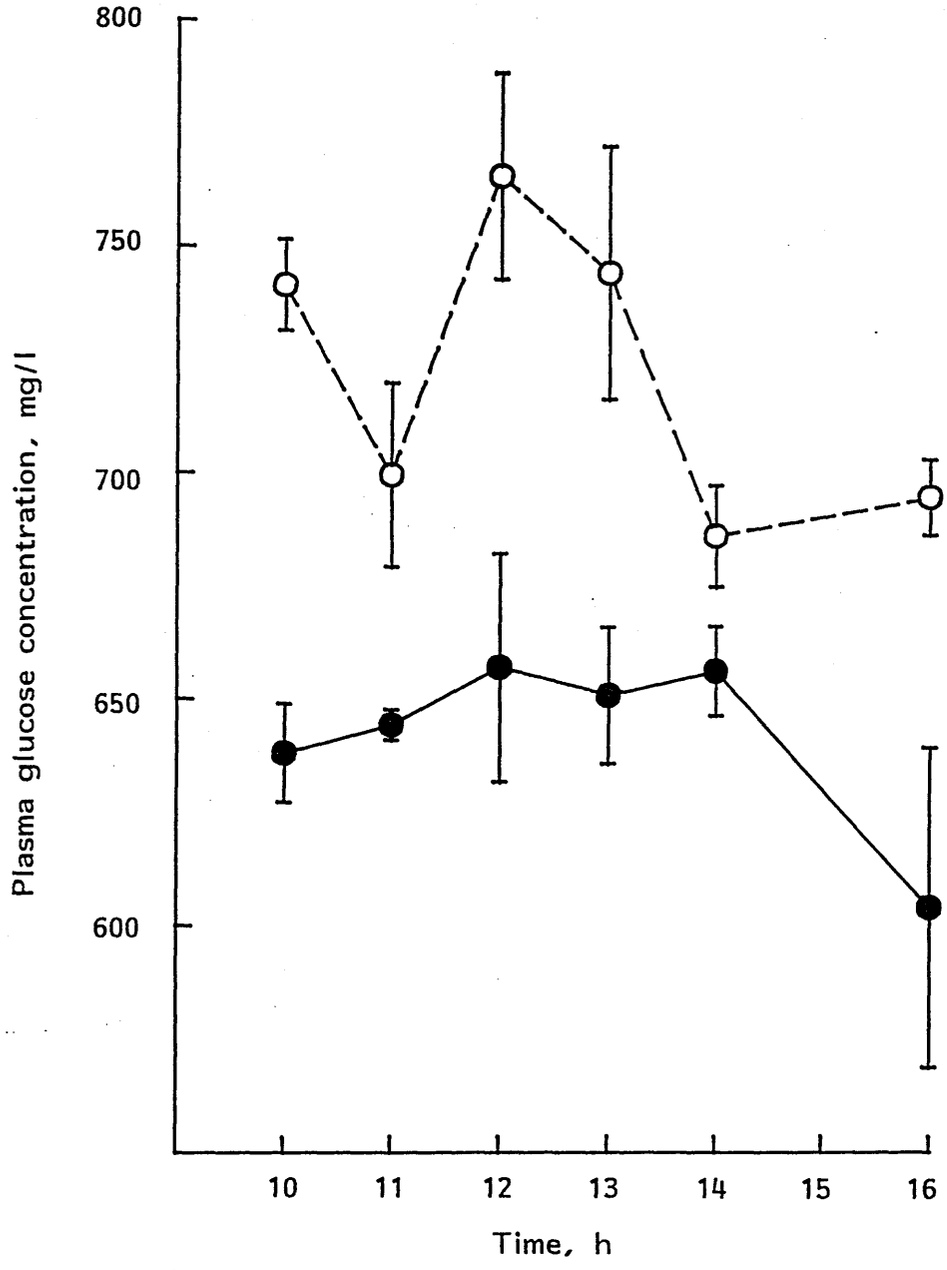


Figure 3.2e Changes during the day of plasma concentration of glucose (mg/l) in cows given 240g urea/d either as a continuous intraruminal infusion (●) or as 2 doses/d (○) at 10.00 and 22.00h

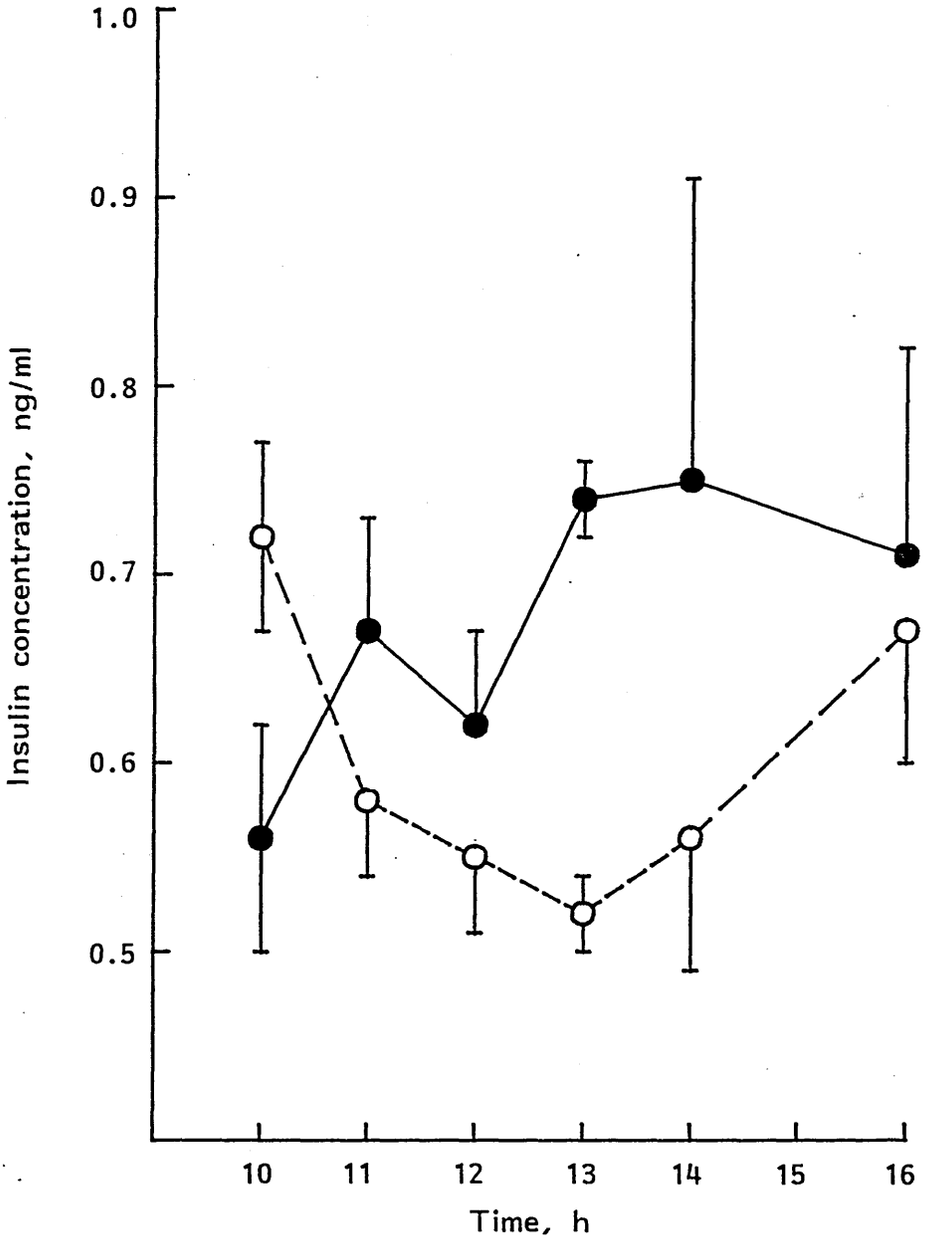


Figure 3.2f Changes during the day of plasma concentration of insulin (ng/ml) in cows given 240g urea/d either as a continuous intraruminal infusion (●) or as 2 doses/d (○) at 10.00 and 22.00h

Table 3.14 The effect of two different methods of intraruminal infusion of urea on the concentrations of plasma free amino acids (μmol/l) in Experiment 4

	Continuous infusion	Twice daily infusion	s.e.d.	
Essential AA				
His	62	61	1.4	
Thr	96	75	2.5	**
Arg	122	111	3.3	*
Trp	44	33	1.5	***
Met	23	22	1.5	
Val	267	228	8.3	**
Phe	65	58	1.7	**
Ile	114	94	3.6	**
Leu	127	115	4.6	*
Lys	108	96	3.1	*
EAA	1028	893	28.8	**
Non-essential AA				
Asp	13	11	0.6	*
Glu	93	76	4.5	**
Ser	59	59	3.4	
Gly	125	139	6.3	
Ala	157	155	5.0	
Tyr	54	46	1.8	**
NEAA	506	485	13.1	
TAA	1534	1378	39.3	*
Intermediate metabolites				
Asn	17	18	1.0	
Gln	262	353	19.3	**
Tau	24	33	1.5	**
α-Aba	10	6	0.4	***
Orn	106	83	3.5	**

3.4 Discussion

In the experiments in which progressively increasing doses of urea were given intraruminally (Experiments 1, 2 and 3), at the highest dose rates the animals did not show any clear signs of ammonia toxicity. However, some individuals showed signs of general unease and restlessness, of a type similar to that described by Symonds *et al.* (1981), which suggests that the highest doses of urea in each experiment were close to the limit beyond which clinical symptoms of toxicity would be expected.

The results of Experiments 1, 2 and 3 in which urea was infused continuously into the rumen, are consistent with the proposal (see Lewis and Buttery, 1972) that ammonia is efficiently extracted by the liver such that urea concentration in peripheral blood increases steadily but ammonia concentrations remain unaffected until the threshold of the liver is exceeded. Plasma urea concentration was linearly increased until NH_3-N levels in the rumen reached approximately 350, 380 and 280 mg/l in Experiments 1, 2 and 3 respectively and its increment was reduced thereafter (Figure 3.3). The plateau in plasma urea concentration was accompanied by increased plasma ammonia and depressions in silage intake and milk production. Lewis, Hill and Annison (1957) reported that peripheral blood ammonia rose when rumen ammonia-N exceeded 84 mg/100ml, but results in Experiments 1, 2 and 3 indicate that the liver is unable to completely detoxify ammonia when rumen ammonia-N concentrations are lower than those proposed by Lewis *et al.* (1957). However, in Experiment 4 when urea was administered twice daily, ammonia concentrations in peripheral blood increased substantially while the increase in plasma urea concentration was relatively small for 2-3 hours following the administration of non-toxic doses of urea (Figure 3.2c). Bartley *et al.* (1981) observed similar trends in cows given 0.5g urea/kg body weight as a single daily dose into the rumen; rumen pH was increased and the concentrations of ammonia in the rumen, portal and jugular blood were rapidly increased 4-9 fold during the first five minutes after dosing but only a small amount of urea appeared in carotid and jugular blood. Bartley *et al.* (1981) and Davidovich *et al.* (1977) suggested that, despite production of urea from ammonia by the liver, ammonia constantly leaks from the liver to the general circulation as indicated by

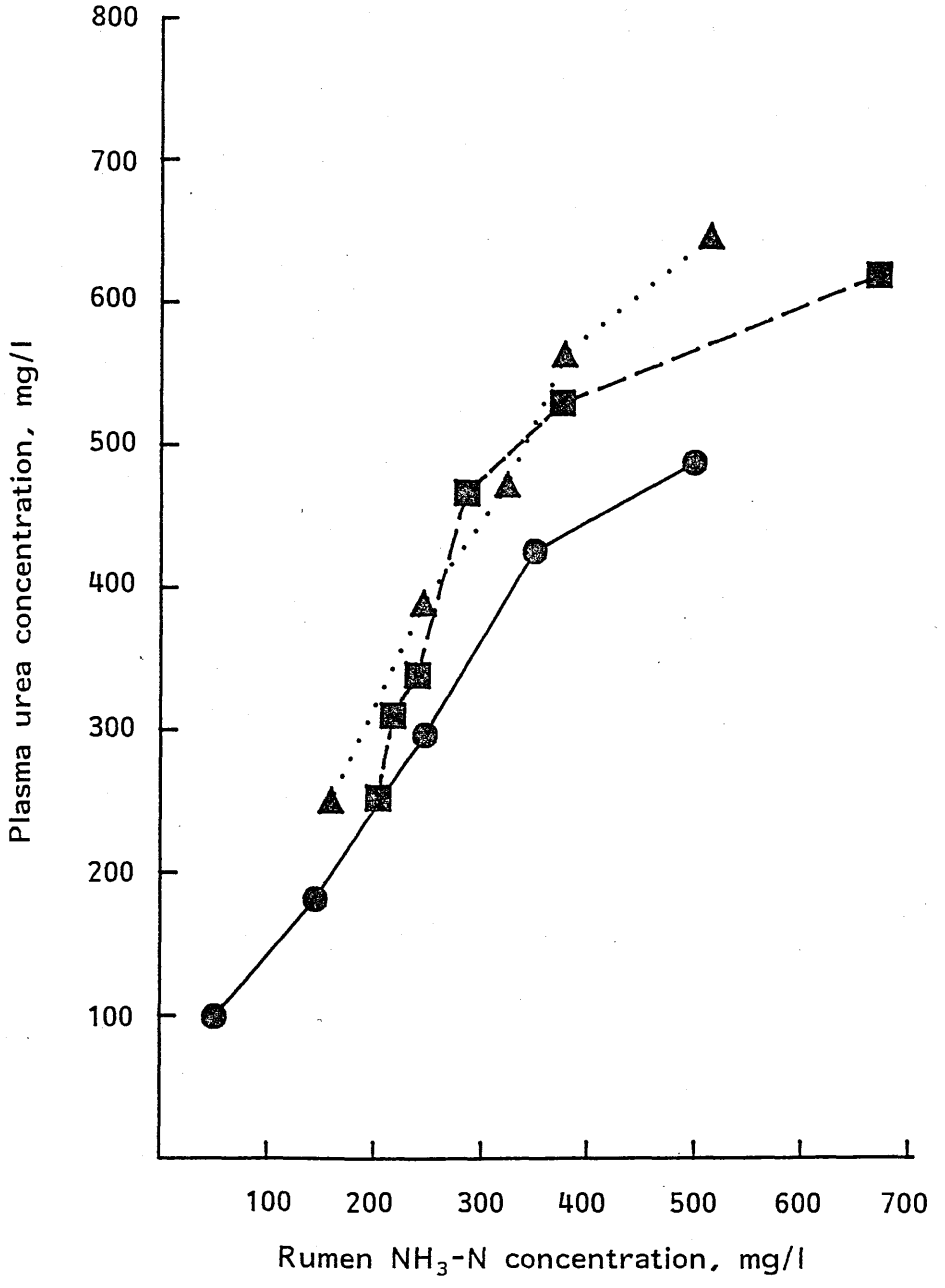


Figure 3.3 Relationships between concentrations of rumen NH_3-N and plasma urea in Experiments 1 (●), 2 (▲) and 3 (■)

the short time taken for ammonia to reach high concentrations in carotid blood. However, the rapid increase in plasma ammonia and concurrent, small increase in plasma urea concentration observed in Experiment 4 may reflect a greater importance of the rate of absorption than the total input in determining the threshold in the liver (Symonds *et al.*, 1981). Ammonia can reach peripheral blood via the thoracic duct so bypassing the liver (Chalmers, Jeffrey and White, 1971), but this does not appear to be a major contributor of ammonia to the peripheral circulation since the movement of lymph to the peritoneal circulation is slow and the ammonia concentration in lymph peaked long after ammonia concentrations in carotid blood (Bartley *et al.*, 1981).

Experiment 3 was designed to examine the effect of rumen pH on the response to urea infusion since more of the ammonia exists as the unionized species at high pH and the lipid layers of the rumen mucosa are more permeable to this than to the NH_4^+ form (Chalupa, 1972). Differences in rumen pH between Experiments 2 and 3 were not as big as planned. Results showed no evidence of effects of rumen pH, within the range 5.9-6.4, as judged from the concentrations of ammonia in peripheral blood, silage intake and milk production, the patterns of response being very similar in the two experiments (Tables 3.10 and 3.12).

The first objective of this series of experiments was to study the influence of ammonia absorption from the rumen on the voluntary intake of grass silage. When urea was infused continuously in progressively increasing dosages, the results show that silage intake was significantly (at least $P<0.05$) reduced at the highest rates of infusion and the depression in silage intake was accompanied by increased concentrations of plasma ammonia (Tables 3.2, 3.5 and 3.10). Taking the results in Experiments 1, 2 and 3 at face value, the implication is that depressions of intake due to ammonia absorption from the rumen are not likely to be encountered in practice. However, when the urea was given in twice-daily doses rather than administered continuously, depression ($P<0.01$) of silage intake was observed even when the diet contained only 170g CP/kg DM (Table 3.13). Similarly, Wilson *et al.* (1975) reported that pouring urea into the rumen twice a day significantly depressed dietary intake below the levels found when urea was infused continuously. The clear suggestion from Experiment 4 is

that when animals consume discrete meals during the day, the susceptibility to intake depression due to ammonia absorption is greatly increased; presumably, the less frequent the meals, the greater the susceptibility. Furthermore, as well as leading to the depression in voluntary intake, diets containing urea can change feeding behavior; smaller and shorter meals (Conrad *et al.*, 1977; Casper and Schingoethe, 1986) and depressed feed intake occurred primarily during the first 30 min after feeding (Wilson *et al.*, 1975). It could be argued that urea does not adequately represent dietary protein since it is degraded to ammonia more rapidly than protein. However, this objection is less serious with respect to the crude protein of grass silage which is present very largely as non-protein nitrogen compounds that are broken down rapidly to ammonia (Thomas and Chamberlain, 1982b). Although further work is necessary before reaching firm conclusions about the implications for practical feeding, taken overall, these results suggest strongly that, in many practical situations, the intake of high-protein silages may be depressed by factors associated with high rates of absorption of ammonia from the rumen.

As regards the amino acid profiles in plasma, results showed different responses depending on whether cows were given silage alone (Experiment 1) or silage plus concentrate (Experiments 2 and 3). In general, infusion of urea decreased the concentrations of most plasma essential amino acids (EAA) in Experiment 1 (Table 3.4) whereas the levels were depressed significantly at the higher doses in Experiments 2 and 3 (Tables 3.7 and 3.11). Presumably, differences in the physiological status of the groups of cows used, non-lactating cows in Experiment 1 versus lactating cows in Experiments 2 and 3, would have contributed, at least partly, to the different responses in amino acid concentrations. Whatever the precise cause for the differences, marked depression of plasma amino acids at the higher doses of urea infusion appears be related to effects of absorbed ammonia on the utilization of amino acids as evidenced by the results of Experiment 4; however, it must be remembered that food intake depressions may have contributed to decreased concentrations of amino acid in plasma. In a series of experiments in which 1.25 mmol ammonium chloride/min was infused into the mesenteric vein of calves to mimic the situation with silage feeding, Wilton (1989) observed a

trend for an increased relative contribution of amino acids to glucose synthesis. Symonds *et al.* (1981) observed increased blood glucose concentrations only when the animals were severely intoxicated but the present results show that increases in the concentration of ammonia in peripheral blood were accompanied by raised blood glucose levels and reduced concentrations of insulin, confirming earlier reports (Barej and Harmeyer, 1979; Fernandez *et al.*, 1988) of an interference with glucose metabolism in ruminants in subclinical stages of ammonia toxicity. The mechanism is still a matter for discussion but it appears that ammonia exerts a direct effect on various hormones, such as insulin, glucagon and catecholamines (Barej and Harmeyer, 1979; Emmanuel *et al.*, 1982; Strombeck, Rogers, and Sterns, 1978), which may also be accompanied by changes of responsiveness of target organs (Barej *et al.*, 1982). Increased blood glucose levels derive mainly from a reduced rate of glucose utilization in insulin-sensitive extrahepatic tissues but there is also an attendant reduction in the production of glucose via gluconeogenesis (Emmanuel and Edjtehadi, 1981; Fernandez, 1987; Spire and Clark, 1979). Krebs, Lund and Stubbs (1975) have suggested that gluconeogenesis and urea synthesis in the liver are interconnected because they share a need for ATP and therefore compete for that source of energy particularly in the presence of high levels of ammonium ions. The fact that both production rate and utilization rate of glucose can be affected, presumably by separate mechanisms, possibly explains the variation in reported effects of urea feeding on blood glucose concentrations (e.g. Leonard, Buttery and Lewis, 1977).

The detrimental effects of ammonia on glucose utilization have particular importance for lactating cows because of lactose synthesis, which requires 60-80% of the available glucose, and is the major regulator of the volume of milk produced (Clark and Davis, 1980). A reduced utilization of glucose by the mammary gland at the highest doses of urea is suggested by decreases in the content and yield of lactose in milk (Tables 3.8 and 3.12). The yield of lactose showed greater proportional reductions relative to protein and fat in the milk and this is particularly marked in Experiment 3 where the proportional reductions in yield were 0.17, 0.07 and -0.19 for lactose, protein (corrected for NPN in milk) and fat respectively; a similar trend was evident in Experiment 2. Whether these observations offer a basis for nutritional

manipulation of lactose yield and hence of the concentrations of fat and, especially, protein in milk deserves attention.

CHAPTER 4

CHAPTER 4 THE EFFECT OF INCREASES IN THE POST-RUMINAL SUPPLY OF PROTEIN ON MILK PRODUCTION AND VOLUNTARY FEED INTAKE OF COWS RECEIVING GRASS SILAGE DIETS

4.1 Introduction

Apart from the detrimental effects of the rapid absorption of substantial amounts of ammonia from the rumen on the voluntary intake of silage and on milk production, which was reported in Chapter 3, the highly degradable nature of silage N has further consequences for its utilization by rumen microorganisms. The combination of high ruminal concentrations of ammonia and a low availability of readily-fermentable carbohydrates with silage diets can result in low rates of microbial protein synthesis. This low efficiency of microbial protein synthesis, in turn, has implications for protein supply to the host animal. Not only is there a low supply of total amino acids to the small intestine but, in addition, the reduced synthesis of bacterial protein results in low concentrations of particular amino acids in duodenal digesta, notably methionine and lysine (Thomas and Chamberlain, 1982a and b; Chamberlain *et al.*, 1986).

It might therefore be expected that cows given silage diets would be responsive to protein supplementation and indeed, in practice, protein supplements can increase silage intake and milk production (Castle, 1982; Rae *et al.*, 1986). The precise mechanisms for these responses are not clear. Stimulatory effects of protein supplementation on silage intake could arise from a ruminal level, by way of increases in ration digestibility (Oldham, 1984) or a faster rate of digestion; also the absorbed products of protein digestion in the rumen or small intestine could contribute to intake control (Chamberlain *et al.*, 1989). Furthermore, increases in silage intake complicate the interpretation of milk production responses to protein supplements because ME supply is increased. However, responses in milk production can occur in the absence of effects on silage intake (Girdler *et al.*, 1988b).

There is a tendency for bigger responses in milk production to fishmeal than to soya bean meal supplementation of grass silage diets (see Chamberlain *et al.*, 1989) but whether these differences in responses relate to differences in the UDP content or to differences in the amino

acid composition of the two proteins is not known. In addition, interpretation of the milk production responses to supplementation with different protein sources in terms of amino acid supply to the small intestine is confounded by uncertainties over the extent of ruminal degradation of protein supplements: for both fishmeal and soya bean meal, the extent of degradation in the rumen varies, not only with source but also with the level of inclusion in the diet (Gill and Beever, 1982; Rooke *et al.*, 1986; Rooke and Armstrong, 1987).

In view of these observations, it was decided to investigate the effect of varying the post-ruminal supply of proteins on the voluntary food intake and milk production of cows receiving grass silage diets. A series of three experiments was conducted using intra-abomasal infusions of different protein sources. The first experiment examined the effects of proteins differing in amino acid composition (casein, soya protein isolate, animal protein and yeast products). Experiment 2 examined responses to increasing levels of two protein sources, casein and soya protein isolate. In Experiment 3, the responses to different forms of casein-N (whole protein and protein hydrolysates) were investigated to throw light on the question of the possible involvement of peptides in eliciting the responses.

4.2 Experimental

4.2.1 Animals and their management and health

A pool of nine lactating Friesian cows fitted with permanent abomasal cannulas was used in four experiments: four cows in Experiments 1a, 1b and 2, and six cows in Experiment 3. The animals were in approximately 26 weeks, 14 weeks, 22 weeks and 10 weeks of lactation when the respective experiments commenced, and average body weights were approximately 539 (range 436-618), 506 (range 500-512), 526 (range 498-562) and 515kg (462-572) in Experiments 1a, 1b, 2 and 3 respectively.

The animals were individually housed in metabolism stalls and milked each day at 06.00 and 16.00h.

During the final period of the second Latin square in Experiment 2, one animal on the basal treatment developed mastitis and was withdrawn from the experiment.

4.2.2 Experimental Diets and Abomasal Infusions and Dietary Additions of Protein

In all experiments, animals were given a diet containing grass silage *ad libitum* and 5 kg/d (fresh weight) of soya-barley concentrate as a basal ration. The proportions of soya and barley in the concentrate were adjusted according to the protein content of the silage. Crude protein content of the basal diet was designed to be 140g/kg DM but actual protein contents of the basal diets, determined from subsamples taken throughout the experiments, were 147, 140, 141 and 132 g/kg for Experiments 1a, 1b, 2 and 3 respectively. The ratio of roughage:concentrate in the basal diet was approximately 70:30 on a DM basis.

The silage used in Experiment 1a was made from a sward of perennial ryegrass (*Lolium perenne*) cut at an early stage of growth with a disc mower. After wilting for 2-4 hours, the grass was harvested with a precision-chop harvester set to give a chop length of approximately 20mm and ensiled without additive in a bunker silo. The silages used in Experiments 1b, 2 and 3 were made by similar procedures to the silage used in Experiment 1a but were ensiled with the addition of Add-Safe (ammonium tri-hydrogen tetraformate 550 g/l; ammonium tri-hydrogen tetra-propionate, 150 g/l; BP Chemicals) at 3 l/t fresh grass. Chemical compositions of the diets are shown in Tables 4.1 and 4.2. The silages were well preserved with low pHs between 3.63 and 3.79, a mean lactic acid content of 105 g/kg DM, a mean ammonia content of 131 g/kg total N and no measurable amounts of butyric acid (Table 4.1).

Abomasal infusions of proteins were conducted continuously for approximately 23 h/d by means of a peristaltic pump. Various amounts of protein sources were infused but the total volume of the infusate was kept constant at 6 l/d. Soluble casein (DM, 935 g/kg; total N, 145.3 g/kg DM), casein enzymatic hydrolysate (DM, 959 g/kg; total N, 133.01 g/kg DM), soya protein isolate (DM, 915 g/kg; and total N, 141.78 g/kg DM) and yeast products, untreated yeast (DM, 949.4 g/kg; total N, 71.12 g/kg DM) and heat-treated yeast (DM, 947.6 g/kg; total N, 70.74 g/kg DM) were used as protein sources for infusion. The amino acid

Table 4.1 Chemical composition of silages

	Exp.1a	Exp.1b	Exp.2	Exp.3
Dry matter, g/kg	236	204	212	213
Organic matter, g/kg DM	925	927	913	921
pH	3.63	3.68	3.67	3.79
Total N, g/kg DM	21.2	23.0	21.0	25.0
True-protein N, g/kg N	251	219	236	244
NH_3-N, g/kg N	106	161	148	106
Water-soluble carbohydrate g/kg DM	11	8	6	8
Lactic acid, g/kg DM	136	118	89	78
VFA, g/kg DM				
Acetic acid	22	13	12	21
Propionic acid	0	0	1	1
Isovaleric acid	0	0	1	2
Ethanol, g/kg DM	23	22	27	39
NDF, g/kg DM	563	571	599	574
ADF, g/kg DM	360	349	366	376
$DOMD^+$, g/kg DM	732	nd	nd	nd

$^+$, measured in 4 sheep at maintenance level of feeding; nd, not determined

Table 4.2 Chemical composition of the concentrate ingredients

	Barley			Soya	
	Exp.1	**Exp.2**	**Exp.3**	**Exp.1a**	**Exp.3**
Dry matter, g/kg	820	803	836	867	845
Organic matter, g/kg DM	977	977	976	926	926
Total N, g/kg DM	20.8	17.0	20.6	78.7	72.2
NDF, g/kg DM	166	190	169	140	160
ADF, g/kg DM	58	61	58	85	104
Starch, g/kg DM	618	631	702	42	63

compositions of the protein sources are shown in Table 4.3. The composition of amino acids in casein and casein hydrolysate were very similar. The concentration of total amino acids in the crude protein was higher for casein than for the other protein sources. Casein was substantially higher in Met, Val, Leu and Lys than AP, SPI (soya protein isolate) or yeast products (UTY and HTY); AP (animal protein) was higher in His and lower in Ile, and SPI was higher in Arg than corresponding values in other protein sources. The contents of total amino acids and histidine in yeast products were lower than for the other protein sources.

Animal protein (AP) containing 500g fishmeal, 300g blood meal and 200g meat and bone meal/kg DM (Palmers Ltd, Peterborough) was used for the dietary addition in Experiment 1a. The rates of disappearance of DM and N from Dacron bags incubated in the rumen are shown in Table 2.1 in Chapter 2 and the changes in amino acid composition after rumen incubation are presented in Table 4.4.

4.2.3 Experimental Design and Procedure

4.2.3.1 Experiment 1

Experiment 1a

The treatments were applied in a sequence according to a 4 x 4 Latin square design with four treatments and four 10-day periods. The experimental treatments were (1) basal diet consisting of grass silage *ad libitum* and 5kg of soya-barley concentrate (85 parts of barley and 15 parts of soya on a fresh weight basis)/d, (2) basal diet plus 1.2 kg/d of AP, (3) basal diet plus abomasal infusion of 230g casein/d and (4) basal diet plus abomasal infusion of 225g SPI/d. The amounts of casein and SPI infused were equivalent to 200g protein/d entering the abomasum.

Experiment 1b

The experimental design was as in Experiment 1a except that different protein sources were used. The four treatments were (1) basal diet containing grass silage *ad libitum* plus 5kg of barley/d, (2) basal plus abomasal infusion of 230g casein/d, (3) basal plus abomasal

Table 4.3 Amino acid composition (g/100g CP) of protein sources

	Protein sources[+]					
	AP	**Casein**	**Casein H**	**SPI**	**UTY**	**HTY**
Essential AA						
His	3.6	2.8	2.9	2.3	1.6	1.7
Thr	3.9	4.8	5.0	4.1	4.7	4.7
Arg	5.0	4.0	3.9	7.2	4.5	4.3
Met	2.0	3.3	3.3	1.5	1.5	1.4
Val	5.9	7.0	7.6	4.7	5.1	4.9
Phe	5.0	5.9	5.5	5.3	4.3	4.1
Ile	2.7	5.5	6.1	4.5	4.3	4.2
Leu	8.8	10.2	9.9	7.8	6.6	6.3
Lys	5.9	8.2	8.3	4.8	5.7	5.5
TEAA	42.8	51.6	52.4	42.2	38.1	37.1
Non-essential AA						
Asp	9.1	7.7	7.9	11.2	9.2	9.1
Glu	6.8	15.3	14.9	11.5	9.9	10.3
Ser	4.3	7.0	6.8	5.6	4.9	4.8
Gly	6.3	2.1	2.1	4.1	4.0	4.0
Ala	6.7	3.4	3.5	4.2	5.5	5.3
Tyr	3.2	6.2	4.7	4.0	3.6	3.6
TNEAA	36.4	41.6	40.0	40.5	37.2	37.1
Total AA	79.2	93.2	92.5	82.7	75.3	74.2

[+]: AP, animal protein; Casein H, casein hydrolysate; SPI, soya protein isolate; UTY, untreated yeast; HTY, heat-treated yeast

Table 4.4 Changes in amino acid composition (g/100g CP) of animal protein (AP) after incubation in the rumen in Dacron bags

	Incubation time, hours						
	0	**8**	**16**	**24**	**48**	**s.e.d.**	
Essential AA							
His	3.9	4.9	4.2	4.2	4.2	0.69	
Thr	4.1	4.7	4.5	4.6	4.6	0.28	
Arg	5.4	5.9	5.6	5.8	5.9	0.35	
Met	2.1	2.4	2.2	2.2	2.2	0.19	
Val	6.4	7.3	6.8	7.1	7.6	0.45	
Phe	5.3	6.7	5.9	6.1	6.4	0.64	
Ile	2.9	3.2	3.0	3.1	3.1	0.24	
Leu	9.5	11.0	10.4	11.1	11.6	0.77	
Lys	6.6	8.7	8.1	7.9	7.8	0.80	
TEAA	46.1	54.6	50.5	52.1	53.3	4.08	
Non-essential AA							
Asp	10.0	10.4	9.9	10.2	10.6	0.78	
Glu	6.9	7.4	7.0	7.2	7.2	0.38	
Ser	4.5	5.0	4.9	4.9	4.9	0.44	
Gly	6.4	6.2	5.7	6.1	6.0	0.13	*
Ala	7.1	7.8	7.4	7.8	7.8	0.47	
Tyr	3.4	3.8	3.6	3.7	3.8	0.19	
TNEAA	37.6	40.5	38.5	39.6	40.4	2.26	
Total AA	83.6	95.1	89.0	91.7	93.7	14.32	

infusion of 585g untreated yeast (UTY)/d and (4) basal plus abomasal infusion of 585g heat-treated yeast (HTY)/d. Again, amounts of protein infused were equivalent to 200g protein. The heat-treated yeast was included because this was of commercial interest to the company that sponsored this part of this work.

4.2.3.2 Experiment 2

The four cows were used in two consecutive 4 x 4 Latin squares separated by one week. In the first square the treatments were (1) basal diet containing grass silage *ad libitum* and 5kg of barley/d, (2) basal plus 110g casein/d infusion into the abomasum, (3) basal diet plus 220g casein/d infusion into the abomasum and (4) basal plus 330g casein/d infusion into the abomasum. In the second square, the treatments were as in the first except that the three protein infusion treatments were 115, 230 and 345g SPI/d. The doses of SPI were designed to be isonitrogenous with the corresponding doses of casein.

4.2.3.3 Experiment 3

The experiment was conducted as a duplicated 3 x 3 Latin square with two blocks of animals. Each block involved 3 animals, 3 treatments and three 10-day periods. The experimental treatments were (1) basal diet plus abomasal infusion of 230g casein/d, (2) basal diet plus infusion of 260g of casein enzymatic hydrolysate/d and (3) basal plus 225g/d SPI infusion. The basal diet consisted of grass silage *ad libitum* and barley at 5 kg/d, and the amounts of infusate were designed to supply the same amount of protein (200g/d) into the abomasum. The enzymatic hydrolysate of casein contained approximately 40-50% of its N in the form of peptides (Technical information, Sigma Chemical Co.).

4.2.3.4 Experimental Procedures

Prior to the start of all the experiments, the animals received the basal diet for an adaptation period of 14 days in which they became established on an intake of silage *ad libitum*. The animals received their ration in two equal meals per day at 06.00 and 16.00h. Feed intake

was measured daily and the amount of silage offered was adjusted to ensure a refusal of approximately 15% of that offered. Milk yield was recorded daily and the composition of milk was determined on a bulked sample of the last four consecutive milkings in each experimental period. Samples of blood were obtained by venepuncture from the tail, and samples were taken at 10.00 and 14.00h on the last two days of each experimental period in all experiments, except Experiment 1a in which blood samples were taken at 10.00, 12.00, 14.00, 16.00 and 17.00h to monitor the changes of metabolites in blood plasma. Body weights were measured on two consecutive days before the beginning of the experiment and at the end of each period.

4.2.4 Chemical Analysis

Minced wet silage was analysed for toluene dry matter, total nitrogen, true protein, ammonia, lactic acid, ethanol, water soluble carbohydrate, VFA and pH. Dried samples of silage were analysed for ash, total nitrogen, NDF and ADF. Concentrate samples were analysed as appropriate for dry matter, total nitrogen, ash, NDF, ADF and starch. Samples of protein sources for infusion were analysed for dry matter, total nitrogen and amino acids. Blood plasma samples were analysed for glucose, urea, NEFA, β-hydroxybutyrate, insulin, growth hormone and amino acids. Milk samples were analysed for total solids, fat, crude protein, lactose and NPN.

4.2.5 Statistical Analysis

The results obtained in all experiments were subjected to analysis of variance using Genstat 5 (Payne *et al.*, 1987).

In Experiment 2, from the practical standpoint of running the trials, it was decided to use two 4 x 4 Latin squares, one for each protein source. Since the same animals were used for each square and there was only a 7-day interval between the two squares, the results were analysed together as one experiment to allow statistical comparisons between the protein sources. Since each level of protein was compared with the control in the appropriate group, not the overall mean control, any period effect will not affect the analysis if it is additive to all

levels of protein. However, if there is a trend that only affects responses to some levels of protein, this may be confounded with the difference between proteins. This should however show as a period effect in each individual square, and no such effect was found to be significant. The ANOVA model used was as follows

$$Y = \text{Mean} + \text{Cow} + \text{Control} + \text{Control.Protein Source} + \text{Control.Protein Level} + \text{Control.Protein Source.Protein Level} + \text{Error}$$

Control has two levels, zero protein and non-zero protein, protein source has two levels, casein and SPI including respective control for each square and protein level has three levels of protein. The treatment effects tested were 'Control' for a difference between the control and a non-zero additive, 'Protein Source' for the difference between protein sources, in relation to their respective controls, 'Protein Level' for the difference between non-zero levels of protein (averaged over both controls) and 'Source.Level' for a difference between protein sources in the shape of response.

4.3 Results

4.3.1 Experiment 1

4.3.1.1 Experiment 1a

Food Intake and Milk Production

The responses in silage intake and milk production to abomasal infusion of different protein sources are shown in Table 4.5. Casein infusion increased silage intake relative to the basal treatment but the effect was not statistically significant ($P>0.05$). Neither AP nor SPI infusion affected silage intake. All three protein supplements increased ($P<0.01$) milk yield compared with the basal treatment. Casein infusion produced more milk than the SPI ($P<0.05$) or AP ($P<0.1>0.05$) treatments; casein infusion led to the biggest increase in milk yield of 2.54 kg/d whilst the corresponding values observed with SPI infusion and the AP supplement were 1.56 and 1.85 kg/d respectively. There was no statistical difference in milk

Table 4.5 The effect of abomasal infusions of different protein sources on feed intake and milk production in Experiment 1a

	Basal	AP	Casein	SPI	s.e.d.	
Silage intake, kg DM/d	10.2	10.0	10.8	9.9	0.42	*
Milk yield, kg/d	16.9	18.8	19.5	18.5	0.49	**
Total solids, g/kg	130	127	126	126	1.8	
g/d	2189	2391	2452	2320	84.6	
Fat, g/kg	42.7	40.7	39.0	39.2	1.6	
g/d	720	755	758	752	43.2	
Protein, g/kg	31.7	32.6	32.2	31.6	0.3	*
g/d	535	610	625	582	18.3	**
Lactose, g/kg	47.9	47.8	48.1	47.4	0.6	
g/d	812	900	934	874	33.0	*
NPN, g/kg	0.24	0.25	0.22	0.23	0.01	*
g/d	3.99	4.72	4.23	4.19	0.23	

yield between SPI infusion and the AP supplement. Neither the concentration nor the yield of milk fat was affected by treatment ($P>0.05$). The concentration of milk protein was higher ($P<0.05$) for the AP supplement than for the basal and the SPI infusion. All three supplements increased ($P<0.01$) the yield of milk protein compared with the basal treatment; casein infusion increased the yield of milk protein relative to SPI ($P<0.05$) but the trend towards a higher protein yield with AP than with SPI was not significant ($P>0.05$). The concentration of milk lactose was not affected by protein supplements and hence responses in lactose yield were similar to those of milk yield.

Although protein supplements did not change the concentration and yield of milk fat, there was a tendency for the content and yield of C_{18} fatty acids in milk fat to increase with protein supplementation (Tables 4.6 and 4.7), but no differences between protein sources were evident. The increases in content and yield of $C_{18:2}$ among the C_{18} fatty acids were significant ($P<0.05$).

Concentrations of Hormones and Metabolites in Blood Plasma

The changes in concentrations of insulin, growth hormone and some metabolites in blood plasma are given in Table 4.8. Although plasma levels of insulin and growth hormone were not significantly affected by treatment ($P>0.05$), casein infusion tended to decrease the concentration of insulin. Among the energy-yielding constituents measured, the concentrations of both glucose and NEFA were higher for SPI than for the casein infusion. Urea levels were increased by all protein supplements and the most marked increase was observed with AP.

The changes in concentrations of plasma free amino acids are shown in Table 4.9. In general, compared with the basal treatment, all protein supplements increased most of the essential amino acids and total essential amino acids (TEAA), and, in general, changes in plasma levels reflected the concentrations in the protein sources (Table 4.4). The changes in concentrations of His and branched chain amino acids, Val, Ile and Leu, were most evident ($P<0.001$, $P<0.05$, $P<0.05$ and $P<0.05$ respectively), whereas the concentrations of Met, Phe and Trp did not change or showed very little change with protein supplementation. There

Table 4.6 The effect of abomasal infusions of different protein sources on fatty acid composition (g/kg) of milk fat in Experiment 1a

	Basal	AP	Casein	SPI	s.e.d.	
$C_{4:0}$	38	39	35	40	2.7	
$C_{6:0}$	25	26	24	26	1.6	
$C_{8:0}$	15	16	16	17	1.3	
$C_{10:0}$	31	32	30	32	2.1	
$C_{12:0}$	39	40	40	41	1.7	
$C_{14:0}$	124	124	127	123	4.8	
$C_{4:0}$-$C_{14:0}$	271	277	273	279	9.8	
$C_{16:0}$	366	340	342	335	8.5	*
$C_{16:1}$	23	31	21	28	2.8	*
$C_{16:0}$-$C_{16:1}$	388	371	363	363	7.7	*
$C_{18:0}$	113	103	119	115	2.8	**
$C_{18:1}$	190	202	205	210	9.9	
$C_{18:2}$	24	32	29	31	2.2	*
$C_{18:3}$	15	16	17	18	2.0	
$C_{18:0}$-$C_{18:3}$	341	352	370	374	14.7	

Table 4.7 The effect of abomasal infusions of different protein sources on fatty acid yield (g/d) in milk in Experiment 1a

	Basal	AP	Casein	SPI	s.e.d.	
$C_{4:0}$	27	29	26	29	2.7	
$C_{6:0}$	18	19	19	19	1.6	
$C_{8:0}$	11	12	13	12	1.2	
$C_{10:0}$	22	24	23	23	2.3	
$C_{12:0}$	28	30	31	29	2.5	
$C_{14:0}$	89	94	97	89	7.7	
$C_{4:0}$-$C_{14:0}$	195	209	208	201	16.3	
$C_{16:0}$	263	257	259	242	17.2	
$C_{16:1}$	16	23	17	20	2.2	*
$C_{16:0}$-$C_{16:1}$	280	280	276	263	18.0	
$C_{18:0}$	81	77	90	83	4.0	
$C_{18:1}$	136	153	154	152	7.6	
$C_{18:2}$	18	24	22	22	1.9	*
$C_{18:3}$	10	12	13	13	1.5	
$C_{18:0}$-$C_{18:3}$	245	266	279	271	13.4	

Table 4.8 The effect of abomasal infusions of different protein sources on the composition of plasma in Experiment 1a[+]

	Basal	AP	Casein	SPI	s.e.d.
Glucose, mg/l	634	636	615	640	12.2
Urea, mg/l	263	333	281	296	23.9
NEFA, mEq/l	0.07	0.10	0.08	0.12	0.02
β-hydroxybutyrate, mg/l	188	192	180	186	10.4
Insulin, ng/ml	0.29	0.33	0.24	0.35	0.09
Growth hormone, ng/ml	3.35	3.29	3.59	3.65	0.35

[+], each value is the mean of five samples obtained over a 8 h period

Table 4.9 The effect of abomasal infusions of different protein sources on the concentrations of plasma free amino acids (μmol/l) in Experiment 1a

	Basal	AP	Casein	SPI	s.e.d.	
Essential AA						
His	21	58	41	45	5.3	***
Thr	100	103	106	106	9.8	
Arg	65	68	74	73	5.8	
Trp	41	40	40	42	2.5	
Met	11	12	13	11	1.3	
Val	141	179	191	157	13.8	*
Phe	46	44	46	46	2.7	
Ile	93	91	115	102	6.7	*
Leu	72	93	99	83	6.4	*
Lys	69	82	82	77	7.4	
TEAA	657	769	806	741	58	
Non-essential AA						
Asp	11	12	10	12	0.9	
Glu	88	81	69	77	10.4	
Ser	96	93	90	104	9.2	
Gly	285	271	263	273	21.9	
Ala	172	183	185	175	15.2	
Tyr	53	49	56	54	4.8	
TNEAA	705	690	672	696	40.3	
Total AA	1362	1459	1478	1436	96.2	

was no significant ($P>0.05$) effect of protein supplements on concentrations of plasma non-essential amino acids (NEAA).

4.3.1.2 Experiment 1b

Food Intake and Milk Production

The effects of abomasal infusion of casein and yeast products on voluntary food intake and milk production are given in Table 4.10. Similarly to the response observed in Experiment 1a, silage intake was increased by casein infusion but the effect was not significant ($P>0.05$). In milk production responses, casein infusion significantly increased the yields of milk ($P<0.01$), milk fat ($P<0.05$), milk protein ($P<0.01$) and milk lactose ($P<0.05$), the increases (% of basal treatment) being 19, 14, 27 and 17% respectively. The concentration of milk protein was increased ($P<0.05$) only by casein infusion. However, neither silage intake nor milk production was improved by abomasal infusions of yeast products.

The composition and yields of milk fatty acid in milk fat are given in Tables 4.11 and 4.12. Casein infusion increased the sum of C_{4-14} fatty acid proportions ($P<0.05$), decreased ($P<0.01$) the proportion of C_{16} fatty acids and there was no change in the concentrations of C_{18} fatty acids. The increases in yield of milk fatty acids with casein infusion were 23, 7 and 18% relative to the basal treatment for $C_{4:0}$-$C_{14:0}$, $C_{16:0}$-$C_{16:1}$ and $C_{18:0}$-$C_{18:3}$ respectively. Of total C_{18} fatty acids, the biggest increase was observed in $C_{18:1}$. Neither of the yeast products showed any effect on the composition and yield of milk fatty acids.

Concentrations of Metabolites in Blood Plasma

The concentrations of plasma glucose, urea, NEFA and β-hydroxybutyrate were not affected by abomasal infusion of the different protein sources (Table 4.13).

The changes of plasma amino acid concentration in response to abomasal infusion of casein and yeast products are shown in Table 4.14. Casein infusion increased the concentrations of total EAA ($P<0.01$) and decreased total NEAA ($P<0.05$) compared with the other treatments. The changes in concentrations of His, Arg, Val, Leu and Lys were

Table 4.10 The effect of abomasal infusions of different protein sources on feed intake and milk production in Experiment 1b

	Basal	Casein	UTY^{+}	HTY^{++}	s.e.d.	
Silage intake, kg DM/d	10.0	10.6	9.9	9.8	0.41	
Milk yield, kg/d	15.5	18.5	15.5	16.1	0.55	**
Total solids, g/kg	134	135	136	132	2.2	
g/d	2090	2490	2105	2120	91.7	*
Fat, g/kg	49.8	48.0	50.3	47.0	2.4	
g/d	774	887	777	756	47.3	
Protein, g/kg	30.1	32.2	30.1	30.3	0.5	*
g/d	466	591	465	486	19.4	**
Lactose, g/kg	46.4	45.6	47.0	46.9	1.0	
g/d	721	845	729	753	36.1	*
NPN, g/kg	0.25	0.25	0.24	0.24	0.01	
g/d	3.83	4.59	3.79	3.83	0.24	*

UTY^{+}, untreated yeast; HTY^{++}, heat-treated yeast

Table 4.11 The effect of abomasal infusions of different protein sources on fatty acid composition (g/kg) of milk fat in Experiment 1b

	Basal	Casein	UTY	HTY	s.e.d.	
$C_{4:0}$	41	36	39	36	1.5	*
$C_{6:0}$	30	30	30	31	1.5	
$C_{8:0}$	22	26	18	22	2.6	
$C_{10:0}$	38	43	40	35	2.2	*
$C_{12:0}$	43	50	42	43	1.7	*
$C_{14:0}$	124	135	122	125	3.1	*
$C_{4:0}$-$C_{14:0}$	298	319	291	290	7.9	*
$C_{16:0}$	385	360	392	382	5.4	**
$C_{16:1}$	31	30	32	35	1.6	
$C_{16:0}$-$C_{16:1}$	416	390	424	417	5.7	**
$C_{18:0}$	110	108	105	108	4.3	
$C_{18:1}$	142	152	152	153	3.2	*
$C_{18:2}$	22	21	20	23	1.8	
$C_{18:3}$	13	10	8	9	1.3	*
$C_{18:0}$-$C_{18:3}$	287	292	285	293	6.2	

Table 4.12 The effect of abomasal infusions of different protein sources on fatty acid yield (g/d) in milk in Experiment 1b

	Basal	Casein	UTY	HTY	s.e.d.	
$C_{4:0}$	32	32	31	27	2.5	
$C_{6:0}$	23	26	23	23	1.9	
$C_{8:0}$	17	22	14	16	2.0	*
$C_{10:0}$	29	38	30	26	3.4	
$C_{12:0}$	33	43	32	32	3.1	*
$C_{14:0}$	94	118	94	93	7.6	*
$C_{4:0}$-$C_{14:0}$	227	278	224	216	18.5	
$C_{16:0}$	303	322	311	293	17.4	
$C_{16:1}$	24	27	25	27	2.2	
$C_{16:0}$-$C_{16:1}$	327	349	336	320	19.2	
$C_{18:0}$	84	98	80	80	5.3	*
$C_{18:1}$	109	135	116	115	7.9	
$C_{18:2}$	17	18	15	17	1.7	
$C_{18:3}$	10	8	6	7	0.9	*
$C_{18:0}$-$C_{18:3}$	221	260	217	220	12.9	*

Table 4.13 The effect of abomasal infusions of different protein sources on the composition of plasma in Experiment 1b

	Basal	**Casein**	**UTY**	**HTY**	**s.e.d.**
Glucose, mg/l	611	619	617	619	13.7
Urea, mg/l	291	278	270	278	18.2
NEFA, mEq/l	0.05	0.05	0.05	0.03	0.01
β-hydroxybutyrate, mg/l	243	251	230	227	15.6

Table 4.14 The effect of abomasal infusions of different protein sources on the concentrations of plasma free amino acids (μmol/l) in Experiment 1b

	Basal	Casein	UTY	HTY	s.e.d.	
Essential AA						
His	14	26	12	15	3.4	*
Thr	125	122	108	123	7.6	
Arg	61	71	60	63	2.5	*
Trp	41	40	38	40	1.7	
Met	19	22	18	20	1.1	
Val	121	167	119	126	9.6	**
Phe	41	45	39	42	2.4	
Ile	84	104	82	84	7.0	
Leu	65	90	56	64	4.5	**
Lys	56	88	55	64	5.0	**
TEAA	625	774	588	640	21.1	**
Non-essential AA						
Asp	15	13	13	16	2.7	
Glu	57	54	56	57	3.8	
Ser	133	119	127	139	7.6	
Gly	495	409	469	444	17.8	*
Ala	155	169	150	161	8.7	
Tyr	50	49	45	48	1.3	*
TNEAA	906	814	860	863	19.3	*
Total AA	1530	1587	1448	1503	41.5	

significant ($P<0.05$, $P<0.05$, $P<0.01$, $P<0.01$ and $P<0.01$ respectively), whereas concentrations of Thr, Trp, Met and Phe did not change or changed little with casein infusion. However, the concentrations of amino acids with infusions of yeast products (UTY and HTY) were lower than those with casein infusion, particularly for His, Arg, Val, Leu and Lys in particular (at least $P<0.05$).

4.3.2 Experiment 2

Food Intake and Milk Production

The effects of increasing levels of casein or SPI infusion on food intake and milk production are shown in Table 4.15. Although silage intake was increased in a non-linear pattern with all doses of casein and SPI, none of the differences were significant ($P>0.05$). There was a progressive increase in milk yield with each level of infusion for both protein sources but, at each level of infusion, increases were greater (at least $P<0.05$) for casein than for corresponding levels of SPI; the highest level of casein infusion increased milk yield by 3.5 kg/d, whereas the corresponding increase for SPI was 1.6 kg/d. The concentration of fat in milk was high throughout and showed no significant differences ($P>0.05$) between treatments. In general, protein infusion tended to increase the concentration of milk protein compared with the basal treatment but this was significant ($P<0.05$) only for the highest level of casein. The concentration of lactose decreased ($P<0.05$) only at the first level of SPI. There were no effects of protein infusion on NPN concentration in milk. In the yield of milk protein, the patterns of response to increasing levels of casein and SPI were different ($P<0.05$). Milk protein output was increased linearly with the level of casein infusion; increases as % of basal treatment being 6, 25 and 37% for 110, 220 and 330 g/d casein infusion respectively. With SPI, however, graded amounts of protein infusion tended to increase milk protein output but differences between infusion levels were small and non-significant; there was no additional increase in the yield of milk protein with the two higher levels of SPI infusion. The yield of lactose was progressively increased with each protein source ($P<0.01$) but the effect was greater with casein. Although the output of milk fat was increased by protein infusion, the

Table 4.15 The effect of abomasal infusions of casein or soya protein isolate (SPI) on feed intake and milk production in Experiment 2

Treatment	Silage Intake kg DM/d	Milk Yield kg/ d	Milk composition, g/kg				Yield, g/d			
			Fat	Protein	Lactose	NPN	Fat	Protein	Lactose	NPN
Basal	9.59	13.9	52.7	31.8	47.3	0.22	725	439	660	3.0
Casein, g/d										
110	9.72	15.7	49.9	30.0	47.3	0.22	779	469	740	3.5
220	9.73	17.1	51.5	32.1	45.3	0.24	879	548	772	4.0
330	10.33	17.4	48.5	34.8	46.0	0.23	834	599	801	4.0
SPI, g/d										
115	10.3	14.5	52.1	33.5	44.9	0.23	756	481	654	3.4
230	9.65	14.9	50.9	33.1	46.2	0.23	749	491	689	3.4
345	10.72	15.5	52.3	32.2	45.9	0.22	811	496	711	3.4
s.e.d.	0.5	0.48	3.18	1.28	0.7	0.14	53.3	26.7	23.9	0.29
Statistical significance										
Control	ns	***	ns	ns	**	ns	*	***	***	**
Protein Source	ns	***	ns	ns	ns	ns	ns	*	***	ns
Protein Level	ns	**	ns	ns	ns	ns	ns	**	**	ns
Source.Level	ns	ns	ns	*	*	ns	ns	*	ns	ns

response was variable.

The composition and yield of fatty acids in milk fat showed a number of statistically significant differences between protein sources and infusion levels but, overall, the differences were small (Tables 4.16 and 4.17). The proportion of total $C_{4:0}$-$C_{14:0}$ acids was greater for casein than for SPI at all levels of infusion. Protein infusion decreased the proportion of total C_{16} acids ($P<0.001$). Total C_{18} acids and most C_{18} acids, except $C_{18:0}$, were higher (at least $P<0.01$) with SPI than with casein infusion. Both protein sources produced similar increases in the yield of total C_{18} fatty acids in milk fat but casein showed greater increases in the output of fatty acids synthesized *de novo* in the mammary gland (Table 4.17).

Concentrations of Metabolites in Blood Plasma

The changes in the concentration of some plasma metabolites are given in Table 4.18. There was no effect of protein infusion on glucose concentration. Urea levels were not affected by SPI infusion but the two higher levels of casein increased urea concentration in plasma ($P<0.05$). At all levels of infusion, casein increased and SPI decreased the concentrations of β-hydroxybutyrate and NEFA relative to the basal treatment, with the result that, at each level of infusion, the value for casein was significantly ($P<0.001$) higher than that for the corresponding level of SPI.

The concentrations of free amino acids in plasma are given in Table 4.19. For both protein sources, the concentration of total essential amino acids increased ($P<0.001$) with a linear trend as the level of infusion was increased but the response for casein was higher than that for the corresponding level of SPI; the increase in TEAA was 19-27% for successive additions of casein and 10-21% for SPI. For some of the essential AA, the changes in concentration with protein infusion were significant (at least $P<0.05$) but, for most of the non-essential AA, there were no significant effects. The concentrations of Thr and Met were higher ($P<0.05$ and $P<0.01$ respectively) with casein than with SPI. With the exception of Thr, Trp, and Phe, the increases in concentrations of His, Met, Val, Ile, Leu and Lys for casein, and concentrations of His, Arg, Val and Leu for SPI infusion were linear with

Table 4.16 The effect of abomasal infusions of casein or soya protein isolate (SPI) on the fatty acid composition (g/kg) of milk fat in Experiment 2

		Casein, g/d			SPI, g/d				Statistical Significance			
	Basal	110	220	330	115	230	345	s.e.d.	Control	Source	Level	Source Level
$C_{4:0}$	44	46	44	41	43	42	42	1.9	ns	*	ns	ns
$C_{6:0}$	27	28	30	28	28	28	27	1.1	ns	ns	ns	ns
$C_{8:0}$	16	17	19	18	17	18	17	0.9	*	ns	ns	ns
$C_{10:0}$	35	35	42	41	40	38	39	3.1	*	ns	ns	ns
$C_{12:0}$	43	41	49	50	47	45	46	3.9	ns	ns	ns	ns
$C_{14:0}$	117	123	130	137	125	124	125	4.0	***	ns	ns	ns
$C_{4:0}$-$C_{14:0}$	282	290	315	316	299	295	296	11.1	ns	ns	ns	ns
$C_{16:0}$	390	388	356	360	360	356	354	10.6	***	ns	*	ns
$C_{16:1}$	25	26	22	25	23	21	23	2.7	ns	ns	ns	ns
$C_{16:0}$-$C_{16:1}$	414	414	378	385	382	377	376	11.2	***	ns	*	ns
$C_{18:0}$	124	115	128	117	124	128	124	6.5	ns	ns	ns	ns
$C_{18:1}$	160	159	157	162	167	176	178	8.1	ns	**	ns	ns
$C_{18:2}$	15	14	13	14	17	17	18	0.9	ns	***	ns	ns
$C_{18:3}$	7	7	7	7	8	9	8	0.5	ns	**	ns	ns
$C_{18:0}$-$C_{18:3}$	306	295	304	300	316	329	327	11.4	ns	**	ns	ns

Table 4.17 The effect of abomasal infusions of casein or soya protein isolate (SPI) on fatty acid yield (g/d) in milk in Experiment 2

	Basal	Casein, g/d 110	220	330	SPI, g/d 115	230	345	s.e.d.	Statistical Significance Control	Source	Level	Source Level
$C_{4:0}$	32	35	39	34	32	32	34	2.3	ns	*	ns	ns
$C_{6:0}$	20	22	26	24	21	21	22	2.0	*	ns	ns	ns
$C_{8:0}$	12	13	17	15	13	13	14	1.4	**	ns	ns	ns
$C_{10:0}$	25	28	37	34	30	29	31	4.1	ns	ns	ns	ns
$C_{12:0}$	31	32	43	41	35	33	37	4.9	ns	ns	ns	ns
$C_{14:0}$	85	96	114	114	94	93	101	8.6	**	ns	ns	ns
$C_{4:0}$-$C_{14:0}$	205	226	276	263	224	221	238	22.2	**	ns	ns	ns
$C_{16:0}$	283	303	312	301	273	268	288	15.6	ns	*	ns	ns
$C_{16:1}$	18	20	19	21	17	16	18	2.3	ns	ns	ns	ns
$C_{16:0}$-$C_{16:1}$	301	323	332	322	290	284	306	16.7	ns	**	ns	ns
$C_{18:0}$	90	89	113	97	94	95	100	10.7	ns	ns	ns	ns
$C_{18:1}$	115	123	138	135	127	131	145	8.2	**	ns	ns	ns
$C_{18:2}$	11	11	11	12	13	13	14	1.0	*	**	ns	ns
$C_{18:3}$	5	5	6	6	6	7	7	0.4	*	ns	*	ns
$C_{18:0}$-$C_{18:3}$	221	229	268	251	240	246	266	19.0	*	ns	ns	ns

Table 4.18 The effect of abomasal infusions of casein or soya protein isolate (SPI) on the composition of plasma in Experiment 2

Treatment	Glucose mg/l	Urea mg/l	NEFA mEq/l	β-hydroxybutyrate mg/l
Basal	671	206	0.08	265
Casein, g/d				
110	671	204	0.11	279
220	669	244	0.11	298
330	657	234	0.12	283
SPI, g/d				
115	710	215	0.07	219
230	699	210	0.07	209
345	674	216	0.07	240
s.e.d.	21.6	16.0	0.01	13.7
Statistical significance				
Control	ns	ns	ns	ns
Protein Source	ns	ns	***	***
Protein Level	ns	ns	ns	ns
Source.Level	ns	ns	ns	ns

Table 4.19 The effect of abomasal infusions of casein or soya protein isolate (SPI) on the concentrations of plasma free amino acids (μmol/l) in Experiment 2

		Casein, g/d			SPI, g/d				Statistical Significance			
	Basal	110	220	330	115	230	345	s.e.d.	Control	Source	Level	Source Level
Essential AA												
His	16	19	26	37	19	26	29	4.9	**	ns	**	ns
Thr	116	133	119	126	96	105	100	14.5	ns	*	ns	ns
Arg	55	68	67	63	68	63	70	5.7	**	ns	ns	ns
Trp	37	39	40	38	38	42	41	3.1	ns	ns	ns	ns
Met	19	20	21	21	22	19	19	1.2	ns	**	ns	ns
Val	121	150	162	172	133	145	157	13.7	***	ns	ns	ns
Phe	43	45	46	42	43	41	42	3.2	ns	ns	ns	ns
Ile	76	98	105	102	90	89	109	11.3	**	ns	ns	ns
Leu	60	74	84	83	72	72	81	8.4	**	ns	ns	ns
Lys	50	59	67	72	71	61	72	7.4	***	ns	ns	ns
TEAA	594	706	736	754	651	663	719	46.2	***	ns	ns	ns
Non-essential AA												
Asp	9	7	8	7	5	5	6	1.8	*	***	ns	ns
Glu	55	51	48	49	53	48	48	4.9	*	ns	ns	ns
Ser	118	120	113	102	116	117	121	12.8	ns	ns	ns	ns
Gly	445	495	445	444	406	476	448	54.4	ns	ns	ns	ns
Ala	152	165	158	167	159	162	157	8.8	ns	ns	ns	ns
Tyr	47	50	51	47	49	44	48	3.2	ns	ns	ns	ns
TNEAA	826	890	824	816	789	852	827	64.3	ns	ns	ns	ns
Total AA	1421	1595	1560	1570	1440	1515	1547	92.1	*	ns	ns	ns

increasing levels of protein infusion.

4.3.3 Experiment 3

Food Intake and Milk Production

The effects of different protein and amino acid sources on silage intake and milk production are shown in Table 4.20. Differences in silage intake were not significant ($P>0.05$). Responses to infusions of casein and casein hydrolysate in most milk production variables, except milk fat, were similar; the hydrolysate of casein increased the concentration and yield of milk fat significantly ($P<0.01$) compared with casein. There was a tendency ($P>0.05$) for higher concentrations and yields of milk protein with both casein treatments than with SPI infusion. Neither the concentrations nor yields of lactose and NPN in the milk were affected by treatment.

The proportions and yields of milk fatty acids are presented in Tables 4.21 and 4.22. Although yield of milk fat was significantly increased by infusions of casein hydrolysate compared with infusions of casein and SPI, there were no differences in milk fatty acid composition between treatments (Table 4.21). In the yield of milk fatty acids, however, infusion of casein hydrolysate markedly increased ($P<0.05$) yields of total $C_{4:0}$-$C_{14:0}$, C_{16} and C_{18} acids compared with the other treatments. Abomasal infusion of SPI also increased the yields of C_{16} and C_{18} fatty acids over casein infusion ($P<0.05$).

Concentrations of Metabolites in Blood Plasma

Changes in concentrations of metabolites in plasma are given in Table 4.23. Although the concentration of metabolites in plasma did not significantly differ ($P>0.05$) between treatments, there was a tendency for increases in the concentrations of glucose with casein, in urea concentrations with SPI and in NEFA levels with casein hydrolysate infusion relative to the other treatments.

Table 4.20 The effect of abomasal infusions of different sources of protein and amino acids on feed intake and milk production in Experiment 3

	Casein	Casein H	SPI	s.e.d.	
Silage intake, kg/d	9.9	9.6	10.0	0.22	
Milk yield, kg/d	20.1	20.2	19.7	0.27	
Total solids, g/kg	126	132	128	1.2	**
g/d	2517	2665	2524	46.8	*
Fat, g/kg	39.6	45.7	41.9	1.2	**
g/d	790	931	827	31.1	**
Protein, g/kg	30.4	30.2	29.8	0.6	
g/d	605	604	582	12.6	
Lactose, g/kg	48.1	48.3	48.7	0.4	
g/d	968	981	964	18.7	
NPN, g/kg	0.23	0.25	0.24	0.01	
g/d	4.61	5.13	4.74	0.19	

Table 4.21 The effect of abomasal infusions of different sources of protein and amino acids on the fatty acid composition (g/kg) of milk fat in Experiment 3

	Casein	Casein H	SPI	s.e.d.	
$C_{4:0}$	29	31	30	0.6	**
$C_{6:0}$	29	29	28	0.3	
$C_{8:0}$	21	20	20	1.3	
$C_{10:0}$	44	40	41	1.9	
$C_{12:0}$	52	49	47	1.5	*
$C_{14:0}$	137	134	134	2.9	
$C_{4:0}$-$C_{14:0}$	311	305	300	5.8	
$C_{16:0}$	330	326	336	6.9	
$C_{16:0}$	33	33	33	1.1	
$C_{16:0}$-$C_{16:1}$	364	359	369	7.0	
$C_{18:0}$	120	125	121	3.0	
$C_{18:1}$	176	183	180	4.8	
$C_{18:2}$	20	22	22	0.9	
$C_{18:3}$	9	10	9	0.5	
$C_{18:0}$-$C_{18:3}$	325	337	331	8.5	

Table 4.22 The effect of abomasal infusions of different sources of protein and amino acids on fatty acid yield (g/d) in milk in Experiment 3

	Casein	Casein H	SPI	s.e.d.	
$C_{4:0}$	23	30	25	1.6	*
$C_{6:0}$	23	26	23	1.1	*
$C_{8:0}$	17	19	16	1.9	
$C_{10:0}$	35	37	32	1.8	
$C_{12:0}$	41	44	37	2.2	
$C_{14:0}$	107	123	109	4.5	*
$C_{4:0}$-$C_{14:0}$	247	278	243	11.7	*
$C_{16:0}$	264	308	283	9.8	*
$C_{16:1}$	26	31	27	1.8	
$C_{16:0}$-$C_{16:1}$	291	339	310	10.9	*
$C_{18:0}$	92	116	99	4.3	**
$C_{18:1}$	138	170	149	7.6	*
$C_{18:2}$	16	19	18	1.5	
$C_{18:3}$	7	9	8	0.7	
$C_{18:0}$-$C_{18:3}$	253	314	274	14.0	*

Table 4.23 The effect of abomasal infusions of different sources of protein and amino acids on the composition of plasma in Experiment 3

	Casein	Casein H	SPI	s.e.d.
Glucose, mg/l	652	631	623	13.4
Urea, mg/l	305	309	321	15.0
NEFA, mEq/l	0.07	0.11	0.07	0.02
β-hydroxybutyrate, mg/l	276	293	271	21.1

The effects of the treatments on plasma free amino acids are shown in Table 4.24. For both casein treatments, concentration of total essential amino acids was similar but significantly ($P<0.01$) higher than that with SPI infusion. There were no significant differences in the concentrations of total NEAA and total AA between treatments. Similarly to the patterns observed in previous experiments (Experiments 1 and 2), the concentrations of Trp, Met and Phe did not change or changed little with experimental treatments. The concentrations of most EAA, except His, Thr and Lys, did not differ between casein and casein hydrolysate; concentrations of His and Thr were significantly ($P<0.05$) higher with casein, and the concentration of Lys was significantly ($P<0.001$) higher with casein hydrolysate. SPI infusion decreased the concentrations of Val and Leu compared with the two casein treatments.

4.4 Discussion

Silage intake was increased with casein infusion in Experiments 1 and 2, and with SPI in Experiment 2 though responses were not dose-related and were statistically non-significant ($P>0.05$). In an investigation of protein-linked responses of silage intake to dietary addition and abomasal infusion of fishmeal, Girdler, Thomas and Chamberlain (unpublished observation, cited by Chamberlain *et al.*, 1989) showed clearly that an intake response can be mediated via an increased supply of protein to the abomasum.

However, in his review on the response to post-ruminal infusion of casein, Clark (1975) observed no effect on intake in most cases in which cows were fed diets containing hay, corn silage and straw, and high proportions of concentrate, whereas Spechter (1972) and Hale and Jacobs (1972) reported increases in intake to casein infusion in cows fed diets containing 1-2.5% urea. Furthermore, when cows receiving either grass silage or pasture were infused with 300g casein, Rogers *et al.* (1979) noted that intake responses were significantly higher with silage than with pasture. These results clearly suggest there is a difference between basal diets in intake responses to increases in the post-ruminal supply of protein. With the diets containing large proportions of highly degradable N either in grass silage or as added urea, protein supply to the small intestine may be inadequate owing to low efficiencies of microbial

Table 4.24 The effect of abomasal infusions of different sources of protein and amino acids on the concentrations of plasma free amino acids (μmol/l) in Experiment 3

	Casein	Casein H	SPI	s.e.d.	
Essential AA					
His	36	29	30	2.1	*
Thr	125	112	108	4.9	*
Arg	71	67	73	2.2	
Trp	45	45	43	1.5	
Met	21	19	17	1.6	
Val	179	180	155	5.1	**
Phe	47	46	46	1.3	
Ile	102	103	98	3.8	
Leu	86	85	79	1.5	**
Lys	62	94	63	6.3	**
TEAA	776	781	713	11.1	**
Non-essential AA					
Asp	11	11	12	0.5	
Glu	48	48	48	0.8	
Ser	101	101	110	2.8	*
Gly	436	435	446	23.4	
Ala	161	165	158	6.5	
Tyr	54	50	49	1.3	*
TNEAA	811	810	822	27.8	
Total AA	1587	1591	1535	28.0	

protein synthesis and this, in turn, could limit the intake by influencing metabolic control mechanisms.

Although reported responses in silage intake to different protein sources in feeding trials are conflicting (see Thomas and Thomas, 1985; Chamberlain *et al.*, 1989), there was a tendency for bigger responses to casein than to SPI in the present experiments: mean increases of 0.43kg vs -0.1kg DM/d for 200g protein supplied post-ruminally either as casein or as SPI in Experiments 1 and 2. A possible explanation for this relates to the differences in amino acid balance between the protein sources, the contents of all essential amino acids except arginine being higher with casein than with SPI. Effects of amino acid balance on intake are not well-defined in ruminants, although there is evidence that imbalance of amino acids and the correction of imbalance affects intake in sheep (Egan and Rogers, 1978).

It thus seems that increased intake of silage in response to post-ruminal supply of protein could arise from the effect of absorbed products of protein digestion on the metabolic control of intake by resetting the digesta load at which 'physical fill' becomes a limiting factor (Egan, 1980; Gill *et al.*, 1988) since there is evidence that, even for highly digestible grass silages, physical 'rumen fill' mechanisms are important in the control of intake (Thomas and Chamberlain, 1982b).

A comparison of the three protein sources using abomasal infusions for all protein sources in Experiment 1 would have been ideal. However, practical problems prevented the infusion of the fishmeal-based product (AP) into the abomasum in Experiment 1a: even though the reservoir holding the suspension of AP was stirred continuously, continual blocking of the infusion lines occurred and the procedure was abandoned. It was decided to use a dietary addition of AP instead, the level of addition being chosen as that which elicited close to maximal responses of milk production in feeding trials with diets similar to that used here (Chamberlain *et al.*, 1989). This makes the interpretation of responses in terms of amino acid supply to the small intestine more difficult for treatment AP since a value has to be assumed for the extent of ruminal degradation of the product. Using the measured rate of disappearance of

nitrogen from AP (Table 2.1) and assuming a rumen outflow rate of $0.08h^{-1}$ (ARC, 1984), the effective degradability of AP was calculated to be about 0.50 and the calculated total amino acid supply to the small intestine from the AP supplement was 353 g/d. Total amino acids supplied by abomasal infusions of casein, SPI and yeast products (UTY and HTY) were approximately 180 g/d.

Although all three protein treatments significantly improved milk production compared with the basal treatment in Experiment 1a (Table 4.5), responses in milk production to AP were disappointing considering the amount of amino acids supplied into the small intestine by this treatment; the response was considerably smaller than that seen with abomasal infusion of low doses of protein even where the infused protein would conventionally be regarded as having an inferior amino acid balance (SPI) to that of the animal protein. The results suggest that either current estimates of the UDP concentration of some protein supplements from Dacron-bag degradabilities are serious overestimates or the mechanism underlying the responses to abomasal infusion of protein differs from that operating when UDP supplements are given via the diet.

The yeast products elicited no responses in either silage intake or milk production compared with the basal treatment. The most obvious explanation for the lack of response would be either a poor amino acid balance of the protein or a low digestibility of the protein in the small intestine which is suggested by the lack of any positive effects on amino acid concentrations in blood plasma (Table 4.14).

The only difference between the protein supplements in milk production in Experiment 1a was the greater ($P<0.05$) increase of milk protein yield for casein compared with SPI; the SPI response was approximately half of that with casein (Table 4.5). Since these two treatments supplied similar amounts of total amino acids to the small intestine and their digestibilities also can be expected to be similar (Beynen *et al.*, 1990), the difference in response seems related to differences in their amino acid compositions. In Experiment 2, in which responses to increasing amounts of protein either with casein or SPI were compared, results showed clear differences ($P<0.05$) in response pattern of milk protein output between the two protein

sources (Figure 4.1). While increasing levels of casein infusion linearly increased milk protein output, no further improvement over the first level was observed with the two highest levels of SPI infusion. This indicates that the different responses were not related to the amounts of total essential amino acids (TEAA) supplied since the highest level of SPI infusion supplied more TEAA and greater amounts of most essential amino acids except Met and Lys than the second level of casein infusion.

If the increases in the output of individual essential amino acids in milk are related to the amounts of EAA absorbed from casein and SPI, assuming a digestibility in the small intestine of 0.85 (ARC, 1984), the values range from 0.17 - 1.04 (Table 4.25). Taking the highest ratio as indicating the limiting amino acid(s), the candidates would be His for casein and Met plus Lys for SPI. A similar calculation for the AP treatment would also implicate Ile. However, such calculations are simplistic. They assume that the incremental responses of milk protein yield are derived solely from the infused protein whereas, in fact, they are derived from the sum of the infused protein and whatever 'surplus' amino acids are available on the basal treatment. In addition, as mentioned earlier, the basis of the calculation can be further confounded by effects of protein supplementation on silage intake. This is illustrated with the casein treatment where the substantial increase of silage intake induces an increase in the supply of amino acids (microbial + undegraded silage protein) to the small intestine of approximately 61 and 75 g/d for the 230g dose of casein in Experiment 1a and the 330g dose of casein in Experiment 2 respectively (ARC,1984).

Whilst caution is needed in their general interpretation (see Bergen, 1979), in controlled experiments such as that reported here, changes of plasma amino acid concentrations in response to changes in amino acid input may give an indication of those amino acids whose supply may limit production responses. When the proteins were supplied post-ruminally, the plasma concentrations of Trp, Met and Phe were not increased or changed very little (Tables 4.9, 4.14 and 4.19) suggesting that they may have been the EAAs in shortest supply relative to demand.

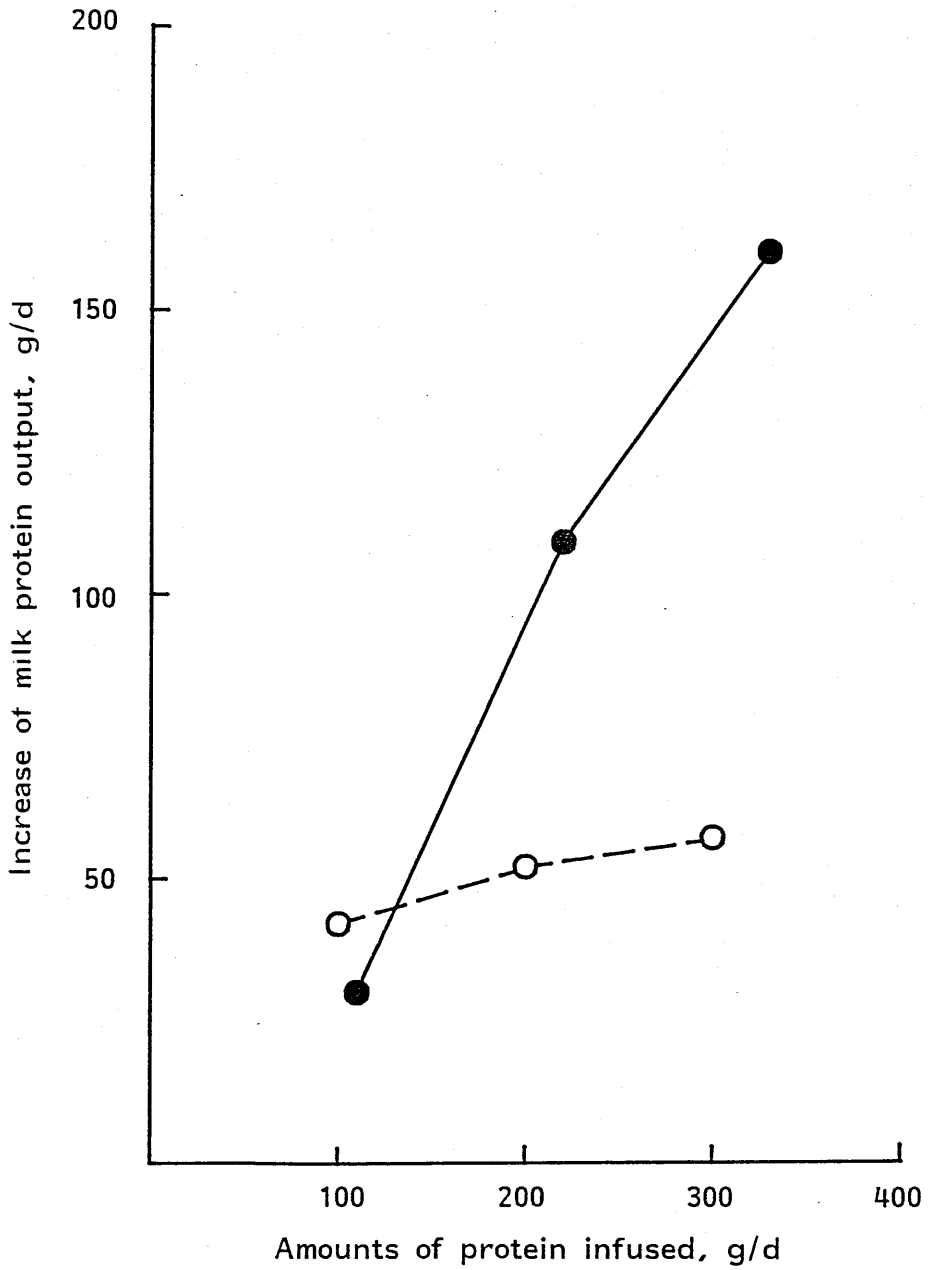


Figure 4.1 Increases of milk protein output in response to abomasal infusion of casein (●) or SPI (○) in Experiment 2

Table 4.25 The inferred apparent efficiency of utilization of absorbed essential amino acids from the infused proteins for milk protein synthesis[+]

	Exp. 1				Exp. 2					
	Exp. 1a			Exp. 1b	Casein, g/d			SPI, g/d		
	Casein	SPI	AP	Casein	110	220	330	115	230	345
His	.64	.39	.19	.98	.48	.89	.88	.68	.42	.31
Thr	.51	.37	.29	.78	.39	.71	.70	.65	.40	.29
Arg	.49	.17	.18	.74	.36	.67	.67	.29	.18	.13
Trp	.49	.42	.39	.75	.37	.68	.67	.74	.45	.34
Met	.44	.60	.33	.67	.33	.60	.60	1.04	.64	.48
Val	.52	.47	.28	.80	.39	.72	.71	.83	.51	.38
Phe	.45	.30	.25	.68	.34	.62	.61	.53	.33	.24
Ile	.50	.37	.47	.76	.37	.69	.68	.65	.40	.30
Leu	.49	.39	.26	.75	.37	.68	.67	.69	.42	.31
Lys	.49	.51	.31	.75	.37	.68	.67	.89	.55	.41

[+], Efficiency of utilization = output of EAA in milk protein/absorbed EAA from infused protein

However, although methionine and lysine have been suggested as limiting amino acids for milk protein synthesis with diets containing grass silage (Thomas and Chamberlain, 1982a; Chamberlain *et al.*, 1986), supplementation of silage diets with methionine or a combination of methionine and lysine intravenously (Chamberlain and Thomas, 1982; Wong, 1984) or in a rumen-protected form or infused intra-abomasally (Girdler *et al.*, 1988a and b) have been singularly unsuccessful in promoting increases in either milk yield or milk protein yield; milk production responses have been limited to inconsistent responses in milk fat content sometimes, but not always, accompanied by increase in fat yield (see Chamberlain *et al.*, 1989). These observations raise the question of whether the observed difference between casein and SPI in the present experiments lies in the suitability of their respective amino acid compositions for synthesis of milk protein, or whether the differences in responses to the two proteins derive from more widely-based effects on metabolism.

In Experiment 2 the cows responded to increasing levels of casein infusion with marked increases in the yields of milk and milk constituents (Table 4.15). In common with earlier reports (Ørskov *et al.*, 1977; Whitelaw *et al.*, 1986), where energy intake in early lactation was restricted to ensure some dependence on body energy stores, increases in the yields of milk protein and lactose were linear. The dose levels of casein in the present experiment covered a lower range than in the other two studies but the highest level of 330 g/d produced an increase in milk protein yield of 37%, substantially in excess of that reported by Whitelaw *et al.* (1986) and compatible with that observed by Ørskov *et al.* (1977) (Figure 4.2). On the other hand, the present study and that of Whitelaw *et al.* (1986) are similar in the observed pattern of response of milk fat output: in both studies, the yield of milk fat reached a maximum with the second level of infusion and was actually decreased by the highest dose level, such that the increase in the energy output in milk reached a 'plateau' at between 10 and 12 MJ/d (Figure 4.2). In contrast, in the experiment of Ørskov *et al.* (1977), the output of milk fat increased progressively with each dose level of casein with the result that the increase in the output of energy in milk with the highest level of casein (750 g/d) was much greater at about 24 MJ/d.

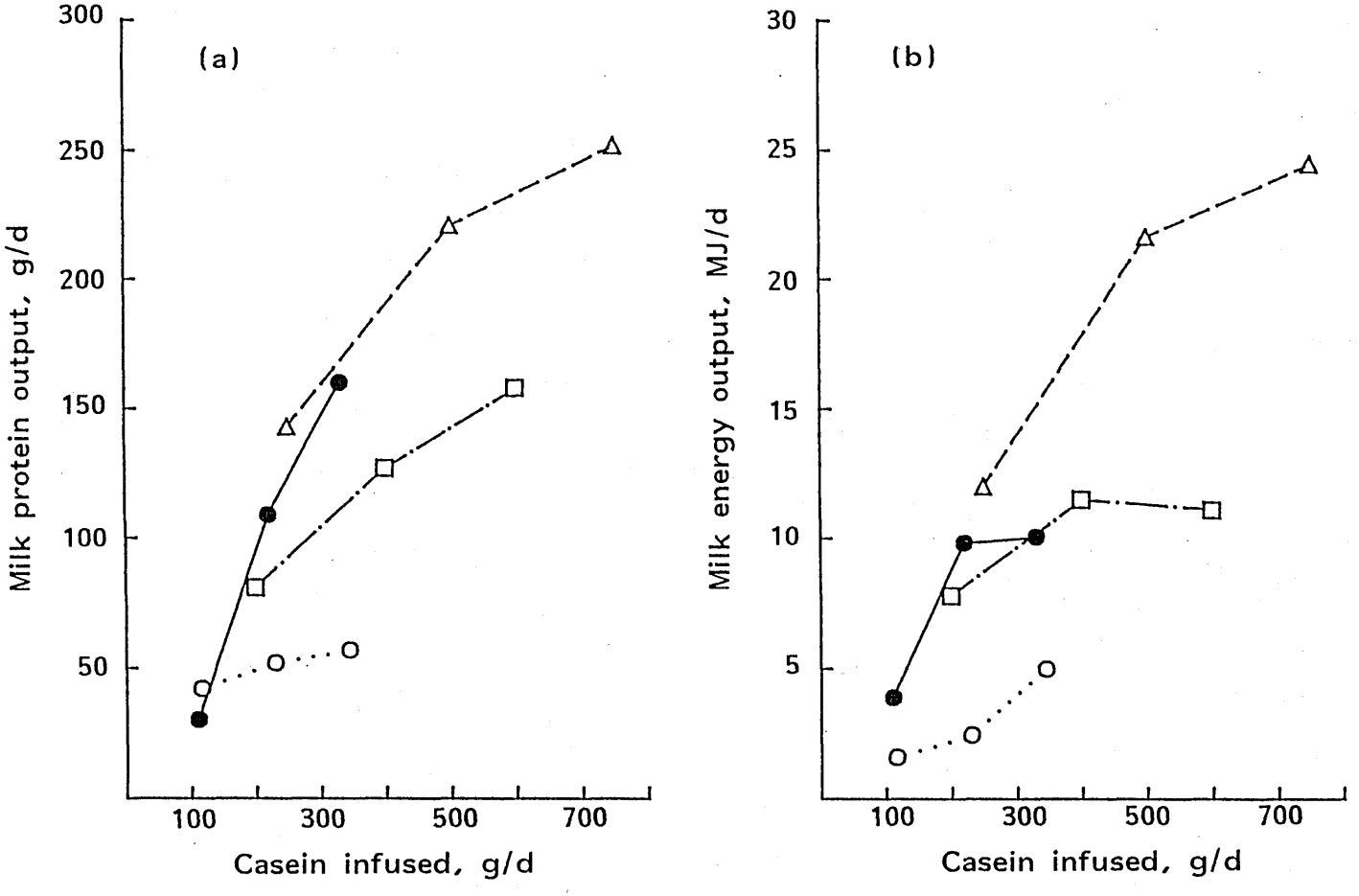


Figure 4.2 The increase in the yields of (a) protein in milk and (b) energy in milk relative to the amounts of casein or SPI infused into the abomasum in dairy cows. Values are from Orskov et al. (1977; △), Whitelaw et al. (1986; □) and the present study (●, casein; ○, SPI).

However, the nutritional and physiological status of the cows in the present experiment contrasted sharply with that of the cows used in experiments of Ørskov *et al.* (1977) and Whitelaw *et al.* (1986). In Experiment 2, animals used were in mid-to-late lactation and were in substantial, positive energy balance on the basal treatment. Calorimetric measurements were not made but an estimate of the energy status of the cows can be deduced. The ME value of the silage can be estimated from its concentration of MAD-fibre (Givens, 1986) to be 10.7 MJ/kg DM. Taking a value of 13 MJ/kg DM for barley, the intake of ME on the basal diet was 155 MJ/d. The ME requirement for maintenance was 50 MJ/d and the efficiency of utilization of ME for lactation (k_l) was 0.63 (ARC, 1980). Using these factors, the ME required to meet the cost of maintenance and the observed output of energy in milk was 128 MJ/d, leaving an excess of 27 MJ/d. Even allowing for the reported low k_l for silage diets by assuming a value of 0.58 (Unsworth, 1990) this still leaves an excess of 20 MJ/d. Although measurements of liveweight changes are subject to error, on average, the animals gained approximately 40kg during the 9-week period of the experiment, thus lending support to the energy balance calculations. Similarly, the supply of protein from the basal diet can be compared with the requirements for maintenance and the observed milk production (ARC, 1984). The supply of rumen-degradable nitrogen (RDN) was 38.6 g/kg OM digested in the rumen (DOMR) which is well in excess of the assumed microbial yield of 30g N/kg DOMR. The total supply of amino acid N to the tissues was 34% in excess of that needed to meet the requirements of maintenance and milk production.

Nevertheless, all three studies have demonstrated that casein infused into the abomasum can increase the output of energy in milk by amounts far greater than the energy supplied by casein itself. However, whereas when the cows are underfed, the extra energy is supplied by mobilized body stores, when the cows are consuming energy and protein in excess of requirement, the extra energy comes either from a repartitioning of nutrient use between body tissues and milk synthesis or from an improved k_l (Oldham, 1984). The biggest effect was seen with the 220 g/d casein level. Assuming a k_l of 0.63 (ARC, 1980) for dietary ME and for the GE supplied in casein, it can be calculated that an extra 12 MJ ME/d was channelled into

milk synthesis, over and above that supplied by the casein itself. The repartitioning of this amount of ME is compatible with the calculation of energy balance made earlier. Alternatively it is possible that the increased energy output was derived from an increase of k_l as suggested by Cowan *et al.* (1981). To account for the effect of casein in this way would require an increase of k_l from 0.50 on the basal treatment to 0.58 with the casein infusion; both values are within the range reported for silage-based diets (Unsworth, 1990). Although the measurements made in this experiment do not allow these two mechanisms to be differentiated, calorimetric studies have failed to show any effect of protein supply on k_l (Tyrrell and Moe, 1980; Trigg *et al.*, 1983; Tyrrell *et al.*, 1983; Vermorel *et al.*, 1983; Whitelaw *et al.*, 1986). On balance then, the effects of casein infusion in this experiment are more likely to derive from effects on the partition of energy between body tissues and milk. If this interpretation is correct, it has important implications for nutrition-endocrine interactions. In cows underfed in early lactation, the effects of casein infusion may be visualized as reinforcing the expected homeorhetic drive in early lactation (Bauman and Currie, 1980) by increasing the mobilization of body tissue to support milk secretion. However, later in lactation, as with the cows in the present experiment, the homeorhetic drive would be expected to begin to encourage the partition of some of the absorbed nutrients into body tissues. These results suggest that, even at this stage of lactation, the homeorhetic mechanism is sufficiently flexible to permit substantial modification of nutrient partition via manipulation of nutrient inputs.

With SPI infusion, however, apart from the first level of infusion, for which increases in the outputs of fat and protein in milk were similar to those with casein, the overall effect on yields of milk and milk constituents were much less than for casein (Table 4.15). Although milk energy output was increased with graded levels of SPI infusion (Figure 4.2), its increase was less than the energy supplied by SPI itself; unlike the case with casein, there was no effect on energy partition. It thus seems that in the circumstances of the present experiment, casein and SPI differed in their effects on the partition of nutrients, which may be related to differences in their amino acid composition or balance. Alternatively, differences between casein and SPI could derive from the rate of absorption of amino acids since, at least in rats,

absorption of amino acids from casein was faster than from SPI (Hara and Knyama, 1991), which may influence endocrine responses and consequently the utilization of nutrients for milk production.

In relation to the effects on energy output in milk, the marked difference between the protein sources in responses of the *de novo* synthesis of fatty acids in the mammary gland (Table 4.17) presumably reflects changes in the pattern of acetate utilization. Indeed, casein infusion increased acetate flux rate in the studies of König *et al.* (1984), lending support to this. Moreover, a stimulation of acetate utilization for milk fat synthesis at the expense of adipose tissue synthesis would be consistent with the observation that casein infusion tended to depress the plasma insulin levels whereas infusion of SPI increased them (Table 4.8).

The possible role of peptides in the casein responses was investigated in Experiment 3. The results showed a pronounced difference in the pattern of response with the casein hydrolysate stimulating the content and yield of milk fat to a much greater extent. However, it would appear that the availability of amino acids from casein and from the hydrolysate used here were similar since responses in milk protein output were identical for the two treatments (Table 4.20) and the results of Experiment 2 show milk protein output to respond linearly and with a high degree of sensitivity to increases in the supply of amino acids from the small intestine. However, it must be remembered that this hydrolysate contained a high proportion (40-50%) of peptides.

CHAPTER 5

CHAPTER 5 AN INVESTIGATION OF (A) THE ROLE OF THE AMINO ACID COMPOSITION OF THE INFUSATE AND (B) THE IMPORTANCE OF THE RELATIVE PROPORTIONS OF PROTEIN, PEPTIDES AND FREE AMINO ACIDS IN THE INFUSATE ON THE RESPONSES OF MILK PRODUCTION AND FOOD INTAKE

5.1 Introduction

In the previous Chapter, in which the responses to abomasal infusions of casein or soya protein isolate (SPI) were examined, results clearly showed superior responses to casein than to SPI in terms of milk production. Differences in the amino acid composition of the respective proteins must be considered as a cause of the superior response for casein. Related to this, the plasma amino acid profiles suggested a potential role for methionine, tryptophan and phenylalanine as limiting amino acids for milk production, and casein is richer in methionine and tryptophan than is SPI.

The results of Experiment 3 in Chapter 4 show clear evidence of differences in responses of milk fat output between casein and a hydrolysate of casein. However, this hydrolysate contained around 40% of its amino acids in peptide form. This raises the question of whether these differences in the pattern of milk production responses would have been greater had the hydrolysate been free of peptides.

Therefore, two further investigations were carried out. The first experiment examined the effects of supplementing SPI with essential amino acids to make it equivalent to casein. A second experiment compared responses to casein with those to a hydrolysate of casein that was virtually free of peptides.

5.2 Experimental

5.2.1 Animals and their management and health

A pool of four lactating Friesian cows fitted with permanent abomasal cannulas were used

in two experiments: four cows in Experiment 1 and three cows in Experiment 2. The animals were in approximately 15 and 20 weeks of lactation when the respective experiments commenced, and average body weights were approximately 495 (range 465-528) and 501kg (range 473-519) in Experiments 1 and 2 respectively.

The animals were individually housed in metabolic stalls and milked each day at 06.00 and 16.00h.

5.2.2 Experimental Diets and Abomasal Infusion

In both experiments, animals were given a diet containing grass silage *ad libitum* and 4 kg/d rolled barley and 1 kg/d soya bean meal as a basal ration. The chemical composition of the dietary ingredients is shown in Table 5.1. The crude protein content of the basal diets was 150 g/kg DM for both experiments and the ratio of roughage:concentrate in the basal diet was approximately 70:30 on a DM basis.

The silage used in both experiments was made from perennial ryegrass (*Lolium perenne*) cut at an early stage of growth and ensiled with the addition of a bacterial inoculant (Ecosyl, ICI plc). The silages were well preserved with low pHs, reasonably low ammonia levels and the virtual absence of butyric acid (Table 5.1).

Abomasal infusions of protein were conducted as in Chapter 4, and casein (DM, 935 g/kg; total N, 145.3 g/kg DM), casein hydrolysate (DM, 816 g/kg; total N, 152.2 g/kg DM) and soya protein isolate (DM, 915 g/kg, total N, 141.78 g/kg DM) were used as sources for infusion. The casein hydrolysate was virtually peptide-free (Technical information, Sigma Chemical Co.).

5.2.3. Experimental Design and Procedure

5.2.3.1 Experiment 1

Four cows were allocated to four columns of a 5 x 5 latin square design with five 7-day periods. The treatments were (1) basal diet, (2) basal diet plus abomasal infusion of 230g casein/d, (3) basal diet plus 273 g/d SPI infusion into the abomasum, (4) basal diet plus

Table 5.1 Chemical composition of the experimental diets

	Silage		Barley		Soya	
	Exp.1	**Exp.2**	**Exp.1**	**Exp.2**	**Exp.1**	**Exp.2**
Dry matter, g/kg	201	204	838	839	843	856
Organic matter, g/kg DM	924	922	976	976	932	932
pH	3.63	3.66	nd	nd	nd	nd
Total N, g/kg DM	22.4	22.1	15.1	15.6	78.1	80.5
True-protein N, g/kg N	291	281	nd	nd	nd	nd
NH_3-N, g/kg N	122	122	nd	nd	nd	nd
Water-soluble carbohydrate g/kg	10	7	nd	nd	nd	nd
Lactic acid, g/kg DM	103	88	nd	nd	nd	nd
VFA, g/kg DM						
Acetic acid	17	18	nd	nd	nd	nd
Propionic acid	2	2	nd	nd	nd	nd
Isobutyric acid	t r	t r	nd	nd	nd	nd
Butyric acid	t r	t r	nd	nd	nd	nd
Ethanol, g/kg DM	10	9	nd	nd	nd	nd
NDF, g/kg DM	627	608	211	197	230	225
ADF, g/kg DM	394	389	68	65	96	105
Starch, g/kg DM	nd	nd	752	723	23	18
$DOMD^+$, g/kg DM	727	727	nd	nd	nd	nd

$^+$, measured in 4 sheep at maintenance level of feeding; tr, trace; nd, not determined

abomasal infusion of 273 g/d SPI plus methionine and tryptophan (SPI + MT) and (5) basal diet plus abomasal infusion of 273 g/d SPI plus a mixture of essential amino acids (SPI + AA). The allocation of cows to treatments was as shown in Table 5.2. The doses of casein and SPI supplied equivalent amounts of total amino acids (184 g/d). Additions of L-amino acids were made on the basis of correcting any deficits in the supplies of methionine and tryptophan (SPI + MT) and of all individual essential amino acids (SPI + AA) in SPI relative to casein. The additions of L-amino acids were: histidine, 0.5g; threonine, 0.4g; tryptophan, 0.6g; methionine, 3.2g; valine, 3.4g; isoleucine, 0.9g; leucine, 2.8g; and lysine, 5.5g. The amino acids supplied by the various abomasal infusions are shown in Table 5.3.

5.2.3.2 Experiment 2

Three cows were allocated to 3 x 8 design with a control period (basal diet) at the beginning and the end of the experiment. Between the control periods each cow had each treatment once, and within periods 2 and 3, 4 and 5 and 6 and 7, each treatment appeared once. Each experimental period was 7 days. Experimental treatments were (1) basal diet, (2) basal diet plus abomasal infusion of 100g casein/d, (3) basal diet plus abomasal infusion of 200g casein/d, (4) basal diet plus abomasal infusion of 400g casein/d, (5) basal diet plus 110g peptide-free casein hydrolysate (PFCH)/d (6) basal diet plus 220g PFCH/d and (7) basal diet plus 440g PFCH/d. The allocation of cows to treatments was as shown in Table 5.4. The doses of peptide-free casein hydrolysate were designed to be isonitrogenous with the corresponding doses of casein.

5.2.3.3 Experimental Procedures

Prior to the start of the experiments, the animals received the basal diet for an adaptation period of 14 days in which they became established on an intake of silage *ad libitum*. Food was given in two equal meals each day at 06.00 and 16.00h. Food intake was measured daily, the silage being given in amounts sufficient to ensure a refusal of about 15% of that offered on the previous day. Milk yield was recorded daily and the composition of milk was determined

Table 5.2 The allocation of cows to treatments in Experiment 1

	Animal No.			
Period	**1**	**2**	**3**	**4**
1	A	B	C	D
2	B	C	D	E
3	D	E	A	B
4	E	A	B	C
5	C	D	E	A

Treatments: A, basal diet; B, basal diet + casein; C, basal deit + SPI; D, basal diet + SPI + methionine + tryptophan; E, basal diet + SPI + mixture of essential amino acids

Table 5.3 The amounts (g/d) of amino acids supplied by the infusion treatments in Experiment 1

	Casein	SPI	SPI + MT	SPI + AA
Essential AA				
His	5.5	5.0	5.0	5.5
Thr	9.3	8.9	8.9	9.3
Arg	7.9	15.8	15.8	15.8
Trp	3.2	2.6	3.2	3.2
Met	6.4	3.2	6.4	6.4
Val	13.6	10.2	10.2	13.6
Phe	11.5	11.7	11.7	11.7
Ile	10.8	9.9	9.9	10.8
Leu	19.8	17.0	17.0	19.8
Lys	16.0	10.5	10.5	16.0
TEAA	104.0	94.8	98.6	112.1
Non-essential AA				
Asp	15.0	24.6	24.6	24.6
Glu	29.8	25.4	25.4	25.4
Ser	13.7	12.3	12.3	12.3
Gly	4.0	8.9	8.9	8.9
Ala	6.5	9.2	9.2	9.2
Tyr	12.2	8.8	8.8	8.8
TNEAA	81.2	89.2	89.2	89.2
Total AA	185.2	184.0	187.8	201.3

Table 5.4 The allocation of cows to treatments in Experiment 2

	Cow No.		
Period	**1**	**2**	**3**
1	basal	basal	basal
2	A	B	C
3	D	E	F
4	B	A	E
5	F	C	D
6	C	F	B
7	E	D	A
8	basal	basal	basal

Treaments: A, basal + 100g casein/d; B, basal + 200g casein/d; C, basal + 400g casein/d; D, basal + 110g peptide-free casein hydrolysate (PFCH)/d; E, basal + 220g PFCH/d; F, basal + 440g PFCH/d

on a bulked sample of the last four consecutive milkings in each experimental period. Samples of blood were obtained by venepuncture from the tail at 10.00 and 14.00h on the last day of each period. Body weights were measured on two consecutive days before the beginning and end of each experiment.

5.2.4 Chemical Analysis

Minced wet silage was analysed for toluene dry matter, total nitrogen, true protein, ammonia, lactic acid, ethanol, water soluble carbohydrate, VFA and pH. Dried samples of silage were analysed for ash, total nitrogen, NDF and ADF. Concentrate samples were analysed as appropriate for dry matter, total nitrogen, ash, NDF, ADF and starch. Samples of protein sources for infusion were analysed for dry matter, total nitrogen and amino acids. Blood plasma samples were analysed for amino acids. Milk samples were analysed for total solids, fat, protein, lactose and NPN.

5.2.5 Statistical Analysis

The results obtained in all experiments were subjected to analysis of variance using Genstat 5 (Payne *et al.*, 1987).

In Experiment 1, since treatment and period effects were not orthogonal, they were estimated sequentially, taking no account of subsequent terms.

The ANOVA model used in Experiment 2 was similar to that in Experiment 2 in Chapter 4, and was as follows

$$Y = \text{Mean} + \text{Cow} + \text{Control} + \text{Control.Source} + \text{Control.Level} + \text{Control.Source.Level} + \text{Error}$$

5.3 Results

5.3.1 Experiment 1

Food Intake and Milk Production

The responses in silage intake and milk production to abomasal infusion of casein, SPI, or SPI with additional amino acids are shown in Table 5.5. Infusion treatments tended to increase silage intake but the differences were not significant ($P>0.05$). All protein infusions increased ($P<0.01$) milk yield relative to the basal treatment. Milk yield was higher ($P<0.05$) for casein than for SPI but differences between casein and the SPI + MT and SPI + AA treatments failed to reach significance ($0.10<P>0.05$). There was a tendency ($0.10<P>0.05$) for higher milk fat content with additions of amino acids (SPI + MT and SPI + AA) and for higher milk protein content with casein relative to the basal treatment. All protein treatments increased ($P<0.001$) the yield of milk protein and tended to increase ($0.10<P>0.05$) the yield of milk fat compared with the basal treatment. Infusion of casein produced a greater milk protein output than all of the SPI-based infusions ($P<0.01$) but differences between the three SPI based treatments were not significant ($P>0.05$).

Concentrations of Amino Acids in Blood Plasma

The changes in concentrations of plasma free amino acids are presented in Table 5.6. None of the differences between treatments reached statistical significance ($P>0.05$) but, in general, the amino acid supplements increased the concentrations of the supplemented amino acids to levels similar to those seen with the casein treatment.

5.3.2 Experiment 2

Food Intake and Milk Production

The effect of increasing levels of casein and its peptide-free hydrolysate on silage intake and milk production are given in Table 5.7. All doses of casein increased ($P<0.05$) silage intake compared with the basal treatment but peptide-free casein hydrolysate (PFCH) increased ($P<0.05$) it only at the highest dose; increases in silage intake were greater ($P<0.05$) for

Table 5.5 The effect of abomasal infusions of casein, SPI, or SPI with additional amino acids on feed intake and milk production in Experiment 1

	Basal	Casein	SPI	SPI + MT	SPI + AA	s.e.d	
Silage intake, kg DM/d	9.6	10.2	9.8	10.1	9.9	0.22	
Milk yield, kg/d	20.2	22.4	21.5	21.9	21.7	0.46	**
Fat, g/kg	43.7	41.2	43.2	44.4	44.9	1.12	
g/d	875	920	926	967	964	32.3	
Protein, g/kg	31.0	32.4	31.3	31.5	31.2	0.43	
g/d	626	725	673	689	691	12.1	***
Lactose, g/kg	47.3	46.2	46.8	46.6	45.9	0.45	
g/d	953	1036	1006	1017	993	22.2	*

Table 5.6 The effect of abomasal infusions of casein, SPI, or SPI with additional amino acids on the concentrations of plasma free amino acids (μmol/l) in Experiment 1

	Basal	**Casein**	**SPI**	**SPI + MT**	**SPI + AA**	**s.e.d.**
Essential AA						
His	9	12	13	12	12	1.1
Thr	163	154	135	146	146	9.4
Arg	63	60	61	67	69	4.7
Trp	44	47	45	48	45	3.6
Met	17	18	15	16	19	1.5
Val	163	189	163	192	179	13.2
Phe	45	44	46	42	46	3.6
Ile	100	109	102	109	114	8.7
Leu	81	88	88	84	91	7.2
Lys	69	74	74	85	76	7.0
TEAA	754	797	741	797	797	51.9
Non-essential AA						
Asp	11	11	14	11	13	3.4
Glu	65	62	60	63	64	3.0
Ser	116	108	121	107	118	12.8
Gly	371	360	344	352	343	30.1
Ala	169	175	159	159	169	13.9
Tyr	50	49	47	41	47	4.8
TNEAA	784	764	746	754	732	49.4
TAA	1538	1561	1486	1529	1552	93.1

Table 5.7 The effect of abomasal infusions of casein or peptide-free casein hydrolysate (PFCH) on feed intake and milk production in Experiment 2

Treatment	Silage intake kg DM/d	Milk yield kg/d	Milk composition, g/kg Fat	Protein	Lactose	Yield, g/d Fat	Protein	Lactose
Basal	9.7	17.7	44.4	31.4	47.8	786	555	847
Casein, g/d								
100	10.0	17.9	42.6	32.3	46.4	766	578	833
200	10.2	18.8	42.4	33.7	46.4	798	632	872
400	10.2	19.5	51.9	33.6	45.8	840	655	896
PFCH, g/d								
110	9.7	17.7	46.8	31.7	47.5	825	559	841
220	9.9	17.0	48.6	33.7	46.5	832	574	790
440	10.0	18.6	43.2	34.0	47.0	804	631	875
s.e.d.	0.14	0.46	1.18	0.49	0.57	19.4	15.4	27.5
Statistical significance								
Control	*	ns	ns	***	*	ns	***	ns
Source	*	*	**	ns	ns	ns	*	ns
Level	ns	*	ns	**	ns	ns	***	ns
Source.Level	ns	ns	ns	ns	ns	ns	ns	ns

casein than for the corresponding levels of hydrolysate at the two lower doses of infusion. There was a progressive increase in milk yield with each level of casein infusion, the effect being significant ($P<0.05$) for the 200 and 400 g/d levels. However, for PFCH only the highest level of infusion increased ($P<0.05$) milk yield. Responses to casein were greater than to equivalent levels of the hydrolysate, the biggest differences ($P<0.05$) between casein and PFCH being observed at the 200g dose/d. There was a tendency, at the first two dose levels, for casein to decrease and for its peptide-free hydrolysate to increase the concentration of milk fat compared with the basal treatment but, for the highest dose rate, this pattern was reversed. In general, both casein and PFCH increased the concentration of milk protein compared with the basal treatment but this was significant ($P<0.001$) only at the two higher doses; there were no significant differences ($P>0.05$) between casein and its hydrolysate. Successive additions of casein linearly increased ($P<0.001$) milk protein output up to 200g casein/d and the slope was lowered thereafter, whereas only the highest dose of hydrolysate increased protein yield. The biggest difference ($P<0.05$) in protein yield between casein and PFCH was observed at the 200 g/d dose.

Concentrations of Amino Acids in Blood Plasma

The changes of plasma amino acid concentrations in response to increasing levels of casein and peptide-free casein hydrolysate are shown in Table 5.8. There were no differences between casein and its peptide-free hydrolysate in the concentrations of either total essential amino acids or individual essential amino acids at the lower doses of infusion. At the highest level of infusion, however, the concentrations of Met, Phe, Leu and Lys in blood plasma were higher (at least $P<0.05$) with the hydrolysate than with the whole protein.

5.4 Discussion

Before discussing the results in details some comments concerning the PFCH need to be made. This product was described as 'virtually peptide free' by the suppliers. However, after completion of the experiments, the material was analysed for peptides by carrying out amino

Table 5.8 The effect of abomasal infusions of casein or peptide-free casein hydrolysate (PFCH) on the concentrations of plasma free amino acids (μmol/l) in Experiment 2

		Casein, g/d			PFCH, g/d				Statistical Significance			
	Basal	100	200	400	110	220	440	s.e.d.	Control	Source	Level	Source Level
Essential AA												
His	15	18	21	53	18	24	40	2.4	***	ns	***	*
Thr	136	127	122	143	105	70	107	9.5	*	***	*	ns
Arg	64	63	61	80	67	62	93	5.5	ns	ns	***	ns
Trp	40	41	39	44	38	38	38	2.6	ns	ns	ns	ns
Met	19	19	17	24	19	20	31	2.3	ns	*	**	ns
Val	155	160	151	245	161	173	293	17.0	**	ns	***	ns
Phe	52	49	47	56	51	50	65	3.5	ns	*	**	ns
Ile	94	94	86	131	90	85	136	10.2	ns	ns	***	ns
Leu	81	88	80	139	101	114	208	18.0	*	*	***	ns
Lys	79	89	94	114	104	102	155	8.1	***	**	***	ns
TEAA	735	748	712	1028	753	738	1165	63.3	*	ns	***	ns
Non-essential AA												
Asp	9	9	6	9	8	7	9	0.9	ns	ns	ns	ns
Glu	98	92	80	85	81	79	94	6.0	*	ns	ns	ns
Ser	107	102	91	102	94	74	87	7.1	ns	*	ns	ns
Gly	357	359	348	320	330	277	279	21.6	*	*	ns	ns
Ala	157	164	150	177	155	158	223	14.3	ns	ns	*	ns
Tyr	43	40	37	53	39	27	39	3.2	ns	**	**	ns
TNEAA	771	765	711	746	706	623	730	46.0	ns	ns	ns	ns
Total AA	1506	1513	1422	1774	1459	1361	1896	128.4	ns	ns	**	ns

acid analysis before and after treating with 6M HCl for 24h at 100°C and attributing the increase in total amino acids following the acid treatment to peptide-bound amino acid residues. By this method, the material contained about 15% of its nitrogen as peptides. Moreover, amino acid analysis revealed an absence of tryptophan in the hydrolysate which, presumably, had been prepared by mild acid hydrolysis. It therefore cannot be ruled out that the absence of tryptophan influenced the results. However, in general, the pattern of response to PFCH resembled those seen with the enzymatic hydrolysate of casein used in the previous chapter.

Consistent with the earlier observations, there was a tendency ($P>0.05$) for bigger responses of silage intake to casein than to SPI in Experiment 1, 0.6 kg vs 0.2 kg DM/d for casein and SPI respectively. The responses to SPI supplemented with amino acids remained lower than for casein (Table 5.5) which may suggest that differences in silage intake between the two protein sources is unlikely to be related to the supply of essential amino acids. However, these differences were statistically non-significant ($P>0.05$).

It can be estimated, assuming a ME concentration of 11.6 (DOMD x 0.16) MJ/kg DM and an efficiency of utilization of ME for lactation of 0.62 (ARC, 1980) that, on the basal treatment in Experiment 1, the cows consumed ME in excess of their requirements for maintenance and milk production (ARC, 1980) by approximately 9 MJ/d. This excess was substantially lower than that in Experiment 2 in Chapter 4 and, unlike previous results, although all infusion treatments increased ($P<0.05$) milk energy output, there was no significant difference ($P>0.05$; s.e.d, 1.61) between treatments; increases in milk energy output were 5.4, 3.9, 5.9 and 5.6 MJ/d for casein, SPI, SPI + MT and SPI + AA respectively. It may be that the responses in this experiment were smaller because of the smaller 'surplus' of ME.

However, in agreement with Experiments 1a and 2 in the previous Chapter, the milk production response to casein was greater than that to SPI. Indeed, in terms of the increases of milk protein yield, all three studies show remarkable agreement: similar levels of casein and SPI infusions increased milk protein output by 90-109 and 47-52 g/d respectively.

Although both of the amino acid additions to SPI (SPI + MT and SPI + AA) produced small numerical increases in the yield of milk protein compared with the unsupplemented SPI treatment, these were not statistically significant ($P>0.05$) and the yields of protein with all three SPI infusions were significantly ($P<0.001$) lower than with casein (Table 5.5). That the milk production response to casein remained superior even when the casein and SPI treatments supplied equivalent amounts of all the essential amino acids into the small intestine (SPI + AA), clearly suggests that differences in responses to casein and SPI cannot be explained solely in terms of their essential amino acid compositions. Since differences in absolute digestibility in the small intestine between casein and SPI are likely to be small (90.1 vs 91.6%; Beynen *et al.*, 1990), other factors must be involved.

Responses of milk protein output to casein and peptide-free casein hydrolysate in Experiment 2 are shown in Figure 5.1. Increases as percentage of the basal treatment being 4, 14 and 18% for 100, 200 and 400 g/d casein, and 1, 3 and 14% for corresponding values of casein hydrolysate respectively. In view of these results, the identical response in the yield of milk protein between casein and casein enzymatic hydrolysate in the previous experiment (Experiment 3 in Chapter 4) may be related to the relatively high content of peptides in that enzymatic hydrolysate of casein. The biggest differences between casein and its peptide-free hydrolysate were observed at the 200 g/d dose; the difference was much smaller at the highest level of infusion.

Again, as in the previous chapter, casein and its hydrolysate had different effects on the concentration and yield of milk fat. The concentration of milk fat was significantly ($P<0.01$) increased by PFCH infusion whereas casein was without effect, the differences between two treatments being most evident at the lower levels of infusion. It is also noteworthy that milk fat concentration was increased with the amino acid additions to SPI in Experiment 1 and has been a common response to amino acid supplementation of silage diets with methionine alone or in combination with lysine. Furthermore, these increases in the concentration and (usually) the yield of milk fat have been observed when supplements of these amino acids have been via the diet, in rumen-protected form (Girdler *et al.*, 1988b), infused intra-abomasally (Girdler *et al.*,

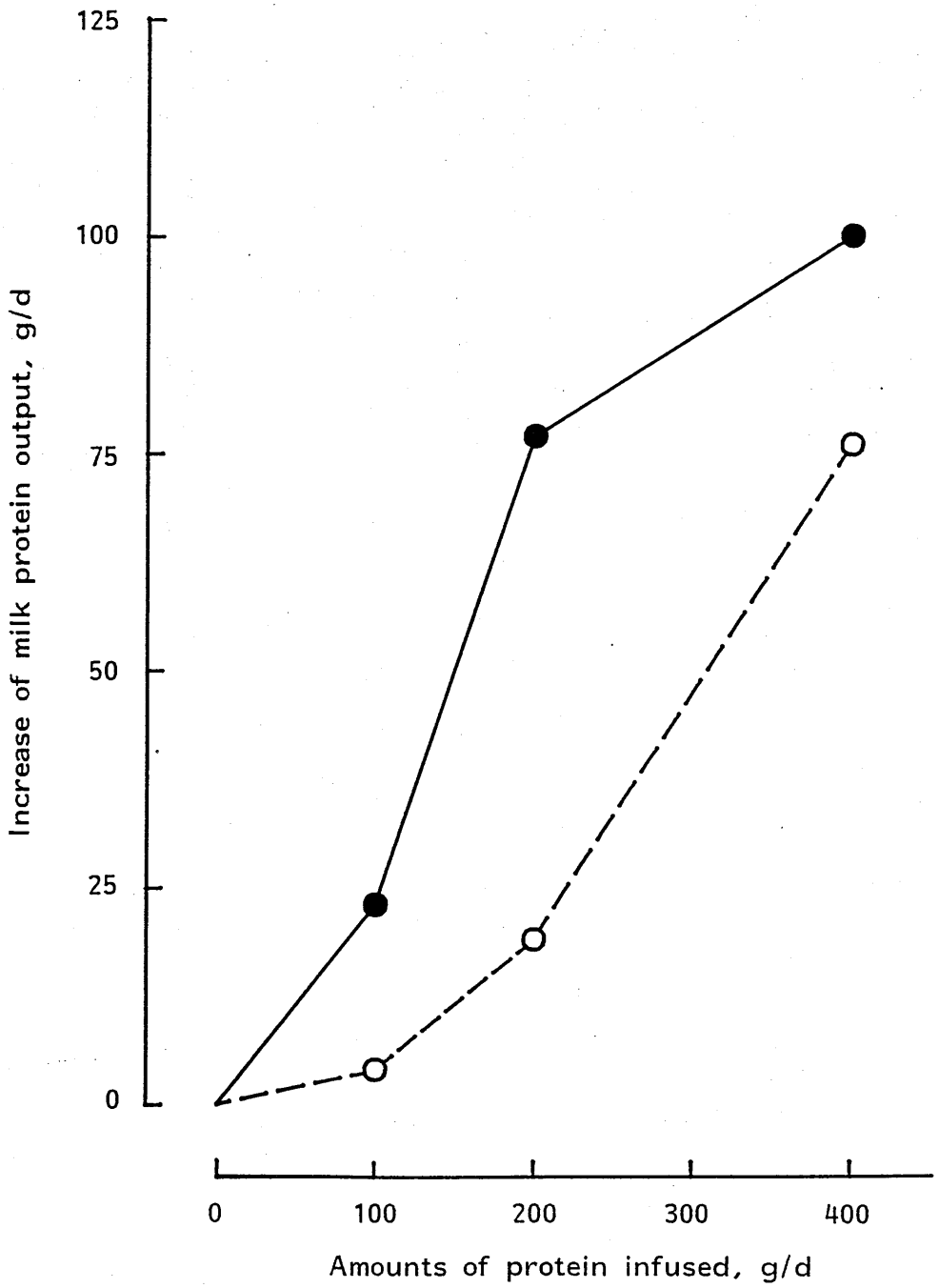


Figure 5.1 Increases of milk protein output in response to abomasal infusion of casein (●) or PFCH (○)

1988a) or infused intravenously (Chamberlain and Thomas, 1982). The mechanism underlying these responses has yet to be uncovered.

It may be argued that the superior response of casein over its hydrolysate may derive from more efficient absorption of amino acids from peptide fractions and whole protein rather than from corresponding mixtures of free amino acids (see Webb, 1990; Webb and Bergman, 1991). Although there is substantial evidence that peptides are absorbed faster over fixed, relatively short time periods than free amino acids (Adibi and Phillips, 1968; Matthews, 1972; Silk *et al.*, 1980), there is no evidence that the net extent of absorption is greater for amino acids given in peptide form rather than as the free amino acids (Rérat *et al.*, 1988; Simoes Nunes *et al.*, 1987). Again, although it is recognized that amino acid concentrations in portal blood are a more reliable index of amino acid absorption (Bergen, 1979), plasma amino acid profiles showed no differences between whole protein and free amino acids at the lower doses of infusion (Table 5.8) even though the biggest difference in milk protein output was observed at the 200 g/d infusion level. In addition, supplementation of SPI with free amino acids raised the concentrations of essential amino acids in peripheral blood plasma to levels similar to those seen with casein infusion in Experiment 1 (Table 5.6).

However, the rate of absorption of amino acids may have influenced endocrine responses and consequently the utilization of nutrients for milk production. In the rat, amino acids were absorbed faster from casein than from SPI (Hara and Kiriyama, 1991). In addition, there is evidence that amino acid absorption from casein was faster than from corresponding mixtures of free amino acids, the largest differences between the two sources occurring during the early stages of absorption (Simoes Nunes *et al.*, 1987; Rérat *et al.*, 1988). Silk *et al.* (1980) reported that amino acids in small peptides from pancreatic hydrolysates of casein, albumin, lysozyme and lactoalbumin were absorbed from rat jejunum more rapidly than equivalent mixtures of amino acids. Hara *et al.* (1984) compared an enzymatic hydrolysate of egg-white protein with a corresponding mixture of free amino acids and found the relative rate of absorption to be 70-80% greater with the peptides during an hour after feeding. This rapid absorption of amino acids can stimulate glucagon release in the pig (Rérat *et al.*, 1988), an

observation which, taken together with the tendency for abomasal infusions of casein to reduce blood levels of insulin relative to those seen with SPI infusion (Experiment 1a in Chapter 4), suggests that the rate of absorption may reduce the molar ratio of insulin to glucagon. Such changes in endocrine secretion would be expected to affect nutrient partition and to favour the partition of nutrients towards the mammary gland at the expense of adipose tissue (see Bassett, 1975 and 1980). In this context, it should be remembered that in Experiment 1 in the present Chapter and in Experiments 1 and 2 in Chapter 4, the animals were consuming energy substantially in excess of their requirements for milk production and were partitioning energy to body tissues. It would, therefore, be expected that there was considerable scope for changes in the partition of nutrients between the mammary gland and adipose tissue in response to hormonal changes.

Some peptides produced during digestion of proteins are biologically active, binding to opioid receptors (Zioudrou, Streaty and Klee, 1979), and this is especially true for casein (Svedberg *et al.*, 1985; Meisel, 1986). Such peptides can affect gut motility and digesta transit time and hence, indirectly, the rate and extent of the digestion and absorption of nutrients but of particular importance is the recent report (Brust *et al.*, 1991) that biologically active peptides (β casomorphins) from casein can bind to opiate receptors in the gut and increase the rate of transport of amino acids across the gut. Moreover, the possibility of such peptides expressing biological activity beyond the level of the gut following absorption into the blood cannot be ruled out (see Gardner and Wood, 1988).

These results suggest that the nutritional value of protein entering the post-ruminal gut of the dairy cow cannot be assessed solely in terms of its amino acid composition or absorbability. Other factors, such as the rate of release of peptides and amino acids during digestion and resultant effects on the rate of absorption of amino acids and the possibility that biologically active peptides may be released during digestion, need to be considered.

CHAPTER 6

CHAPTER 6 GENERAL DISCUSSION

A series of experiments was conducted to establish the importance of aspects of nitrogen metabolism for the control of the voluntary intake of silage and its utilization for milk production. The observations have been discussed at the end of each Chapter but there remains a need for a more integrated discussion of the findings and their implications for the protein nutrition of dairy cows fed grass silage.

Feed protein ingested by ruminants is generally divided into the rumen degradable protein (RDP) and undegradable dietary protein (UDP). The current protein rationing system in the UK (ARC, 1980 and 1984) is based on the concept of RDP as a source of nitrogen to sustain maximum microbial growth in the rumen, and UDP as a source of extra protein to supplement the amino acid N of microbial origin to meet the amino acid N needs of tissues for maintenance, tissue growth, pregnancy and lactation.

6.1 Rumen Degradable Protein (RDP)

Results in Chapter 3, in which urea was infused into the rumen, showed that the voluntary intake of silage may be depressed by factors associated with high rates of absorption of ammonia from the rumen. The use of urea can be criticized in as much as urea does not adequately represent dietary protein since it is degraded to NH_3 more rapidly than protein. However, although the use of urea may exaggerate the effects to some extent, this objection is less serious with respect to the crude protein of grass silage which is present very largely as NPN compounds (40-70% of total N) that are broken down rapidly to NH_3 (see Thomas and Chamberlain, 1982a). The implication of the urea infusion experiments (Chapter 3) is that a high CP content in silage may be an important factor in controlling intake.

Obviously, further work is needed before making practical recommendations concerning optimal CP concentrations in grass silage but the suggestion from these results is that silage intake could be adversely affected when CP concentrations exceed around 170 g/kg DM when

cows receive silage in two meals a day. On the other hand, when urea was infused continuously throughout the day, the critical CP concentrations were higher, being in excess of 200g CP/kg DM. The critical CP concentration in practice may lie somewhere between these two figures and, as such, may be a comparatively rare occurrence.

It is noteworthy also that the lower levels of infusion of urea were sometimes accompanied by increases of silage intake particularly with cows in the early stages of lactation. These responses of silage intake may reflect sub-optimal supplies of ammonia in the rumen despite the fact that the diets supplied RDP in excess of requirements (ARC, 1984). It may be that although feeds such a grass silage contain relatively large amounts of RDP, the rapid degradation of this RDP to ammonia and its subsequent absorption from the rumen may result in deficiencies in ammonia supply in the rumen at times during the feeding cycle. It is interesting that Oldham (1987) has argued that the amounts of dietary CP needed to optimize ruminal digestion of fibre in dairy cows appear to be much higher than ARC (1984) would recommend.

6.2 Undegraded Dietary Protein (UDP)

The ARC (1984) system makes no allowance for different nutritive values of UDP from different protein sources although it recognizes that, as more information becomes available, there may be a need to consider requirements in terms of supplies of essential amino acids (EAA). The failure of the addition of essential amino acids to SPI to induce responses of milk production equivalent to those seen with casein (Experiment 1 in Chapter 5) suggests that there may be a need for an even more sophisticated protein rationing scheme than envisaged in ARC (1984). The implication is that, at least for casein, there are products of protein digestion other than amino acids that need to be considered if the basis of protein responses of dairy cows is to be understood.

It is true that this experiment considered only the supply of EAA. The possible involvement of non-essential amino acids (NEAA) must be considered. Casein is somewhat higher in the concentrations of Glu, Ser and Tyr than is SPI. The role of Glu and Asp has

been examined in abomasal infusion studies with dairy cows but no responses in milk production were obtained (Oldham *et al.*, 1984). One NEAA that has not been considered in the experiments reported here is Pro. The reason for this is that its detection and measurement with the analytical system used here are problematic. However, casein is considerably richer in Pro than is SPI (10% vs 5% of protein; Huff, Hamilton and Carroll, 1977) and the recent demonstration (Bruckental *et al.*, 1991) of positive responses of milk protein yield to the duodenal infusion of Pro may indicate a potential role for this amino acid although it must be said that the very high amounts of Pro infused (80 g/d) makes it difficult to assess the true nutritional or physiological significance of this observation. But what is perhaps more important to the present discussion are the clear differences in response to casein and to hydrolysates of casein (Experiment 3 in Chapter 4 and Experiment 2 in Chapter 5). These results serve as clear demonstrations of the nutritional importance of the form in which amino acids are presented i.e. the relative proportions of free amino acids and peptides.

That these differences in response are due to differences in the availability of amino acids from casein and its hydrolysates seems unlikely in view of the identical responses in milk protein yield in Experiment 3 (Chapter 4). Nor is there any published evidence to support the view that the availability of amino acids from peptides is greater than from corresponding mixtures of free amino acids. However, over fixed time periods, there is evidence of a faster rate of amino acid appearance in portal blood from peptides than from mixtures of free amino acids (Rérat *et al.*, 1988; Simoes Nunes *et al.*, 1987) and also evidence from *in vitro* experiments of faster passage of amino acids supplied in peptides across the gut in everted sac preparations (Burston, Addison and Matthews, 1972; Cheng *et al.*, 1971). Furthermore, the faster rates of amino acid absorption have been accompanied by increases in glucagon levels in plasma (Rérat *et al.*, 1988) in pigs. If similar effects were operating in the lactating cows, then the subsequent reduction of the insulin/glucagon ratio might be expected to favour a repartitioning of energy utilization towards the mammary gland at the expense of body tissues (Bassett, 1975). However, it should be remembered that where these effects of amino acid absorption rate on hormonal balance have been demonstrated, the experimental circumstances

were quite different, in some important respects, from those pertaining here. In the experiments with pigs (Rérat *et al.*, 1988) the animals were fasted before receiving duodenal infusions of casein hydrolysate (rich in peptides) or a corresponding mixture of free amino acids. Hence the infusates represented virtually all the nutrients entering the small intestine (except for endogenous secretions). This contrasts with the present experiments where the animals received a basal diet which provided far and away more absorbed amino acids than did the infusates. It can be calculated, using data from digestion experiments with a similar diet (Chamberlain *et al.*, 1986) and an absorption of amino acids in the small intestine of 0.85 (ARC, 1984), that the basal diet supplied around 1100 g/d of absorbed amino acids. Infusion of 230 g/d of casein could increase this to no more than 1300 g/d. A simple calculation yields figures for the average rate of absorption of amino acids over 24h of 46 and 54 g/h respectively for the two treatments. The question has to be asked whether a difference in the rate of absorption of amino acids from the infused protein only could be expected to influence hormonal balance in these experiments. Clearly, this must remain a possibility: somewhere between 46 and 54 g/h might lie some critical value. On balance, however, this does not seem likely.

On this view, that the differences in response are due to the involvement of peptides in a general sense seems unlikely. This leads on to the possible involvement of specific peptides released during enzymic degradation of casein. The demonstrated physiological effects of casomorphins and other biologically-active peptides derived from casein are of interest here. Apart from effects on gut motility and digesta transit time which could, indirectly, influence the rate of absorption of amino acids, of particular interest is the demonstration that some β-casomorphins can bind to opiate receptors in the gut and directly increase the rate of amino acid absorption (Brust *et al.*, 1991). Such a mechanism could alter the rate and pattern of absorption of amino acids from the total supply (infused + basal) and would appear more likely to bring about alterations of hormonal balance.

The present results identify a role for peptides in milk production responses to the abomasal infusion of casein. This raises the issue of whether these peptide-linked effects occur

more generally with other protein sources. The interaction of protein structure and enzyme specificity will determine the pattern of release of peptides and amino acids during digestion. Casein is very readily digested by pepsin and pancreatic proteases and it is arguable that these peptide-linked effects may be less important with proteins that are less readily digested. However, an investigation of the involvement of peptides in responses to other protein sources should be a priority for future studies. Certainly, it is noteworthy that other proteins, such as gluten, release bioactive peptides during its digestion *in vivo* (Morely *et al.*, 1983).

The increases of silage intake that often accompany protein supplementation of silage diets are difficult to predict (Chamberlain *et al.*, 1989). Again, with the abomasal infusion experiments used here, intake responses were unpredictable in the sense that statistically significant effects were not always observed. In general, there was a tendency for bigger responses to casein than to SPI but the magnitude of the responses varied between experiments presumably as a result of differences between experiments in silage composition and stage of lactation of the cows. Further studies are required to uncover the mechanisms underlying these intake responses and these might be better carried out with longer experimental periods since the question remains as to whether the increases in intake drive the increases of milk production or are a consequence of the increased milk output.

The responses to protein reported here serve to reinforce the comments of others (e.g. MacRae *et al.*, 1988) concerning the need for rationing systems to consider the integration of 'energy' and 'protein' metabolism. Clearly, alterrations of protein/peptide/amino acid supply can have marked effects on the pattern of utilization of energy-yielding substrates for milk production and tissue deposition. Prediction of such interactions is beyond the scope of current protein rationing systems.

In summary, these studies provide some insights into the mechanism of responses of the silage-fed dairy cow to abomasal infusions of casein which conflict with some traditional interpretations of the responses based solely on the supply and demand for amino acids for milk protein synthesis. To what extent these mechanisms are involved more generally, with other dietary protein and other basal diets, must await further research.

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