Chapter 1 Introduction

1.1 General

The efficiency of grazing ruminants, as producers of meat, milk, and wool depends on the quantity and quality of the forage available. As a source of nutrients, forage has variable compositions which are affected by forage species, stage of growth, soil fertility and management factors. Efficient use of forage can only be achieved by understanding the above mentioned factors.

Forage protein is often used inefficiently by animals and unused nitrogenous compounds are present in faeces and urine. Ryden *et al.* (1984) proposed that up to 90% of the N ingested by grazing ruminants is excreted. Under practical farming conditions excreted N can surpass the plant uptake and can contaminate soil and under-ground water supplies (Leng and Nolan 1984).

Ruminant nutritionists are interested in studying protein metabolism in order to maximise protein utilization and increase protein passage from the rumen to the small intestine. On the other hand, there is an awareness of the effects that intensive farming can have on the environment and more attention on how to minimise the amount of N excreted is required. Figure 1-1 shows that nitrogenous wastes from animals play an important role in the N cycle and are also important sources of contaminantion of soil and water resources. Animal waste constituents such as organic matter, nutrients, and fecal bacteria can impact surface water and may also raise the quantity of suspended material in the water and affect the color. Discoloration happens either directly by the waste itself or indirectly through the production of algae and when sediment enters streams from feedlots or overgrazed

pastures. The impact of these contaminants on the aquatic environment depends on type and quantity of each pollutant entering the system and the characteristics of the receiving water. There is limited information on the role of these contaminants in the literature. In fresh forages a considerable fraction of the N is present as non-protein nitrogen (NPN). Much of this NPN is converted to ammonia in the rumen and will be excreted via urine and wasted if it is not efficiently utilised by rumen microbes.

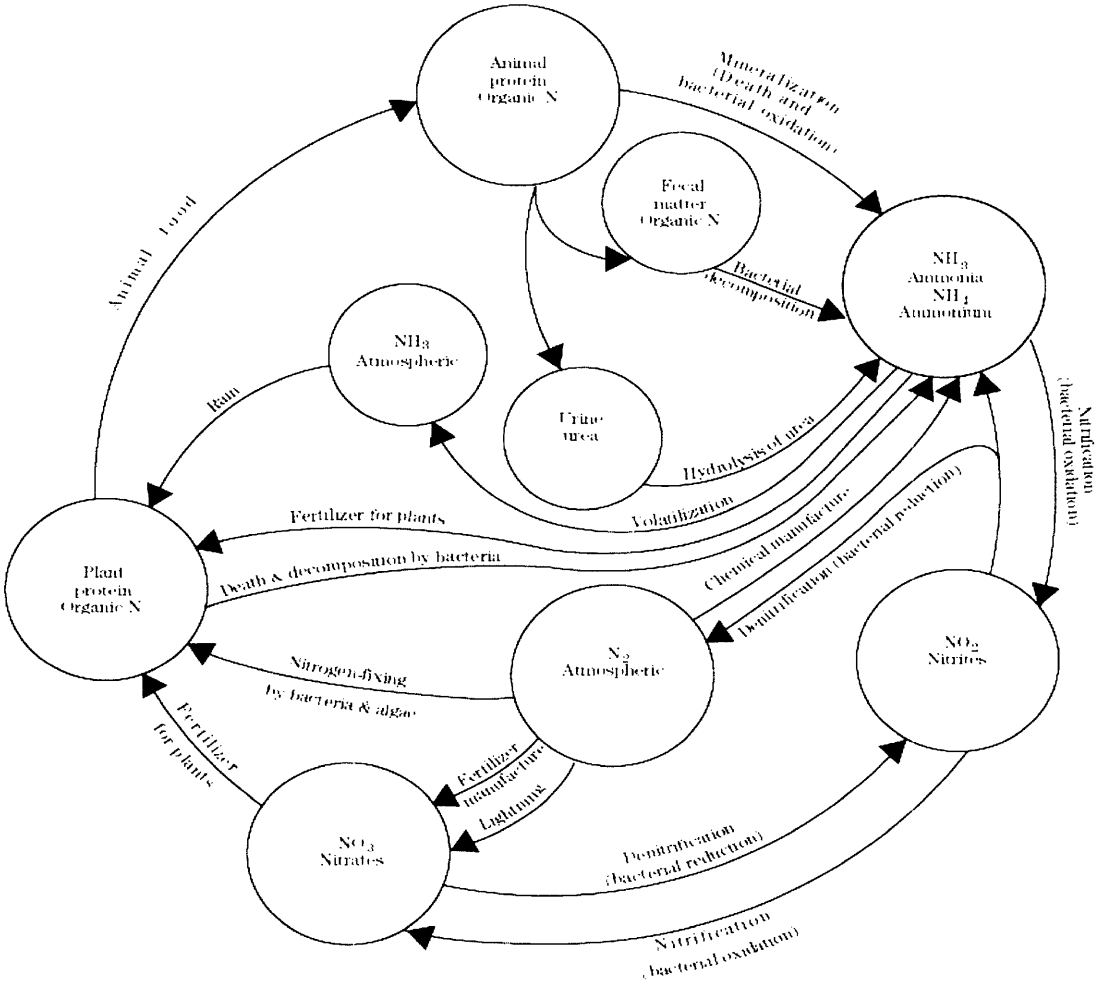


Figure 1-1. The nitrogen cycle (adapted from Walter *et al.* 1999)

1.2 The thesis concept

The studies reported in this thesis were conducted to determine the degradation characteristics of fresh forage DM and protein in the rumen of grazing livestock. Fresh lucerne (a legume) and ryegrass (a grass) were chosen to represent two different species of forages that are commonly grazed by ruminants on improved pastures in Australia. The potential value of these and similar forages as protein sources for ruminants depends their protein degradability in the rumen and ability to support efficient microbial growth or to supply bypass protein to the small intestine. The potential value may also depend on whether peptides or other nitrogenous intermediates are made available for growth of micro-organisms in the rumen. These ideas are investigated further in the experiments reported later in this thesis.

In most forage evaluations, however, instead of fresh samples, dried or frozen-thawed materials have been used (Coblentz *et al.* 1999; Lee *et al.* 2002). The experiments reported in this thesis were undertaken to test the null hypotheses that there is no difference between lucerne and ryegrass and between fresh forage and frozen-thawed forage in terms of degradability characteristics and metabolism in the rumen of sheep. To achieve this aim, experiments using ^{15}N tracer techniques (Carro and Miller 2002; Chapman and Norton 1984; Havassy *et al.* 1982; Hristov *et al.* 2001) were conducted. The ^{15}N-labelled nitrogenous compounds including soluble proteins, peptides and amino acids, ammonia and bacterial protein formed in the rumen from degradation of ^{15}N-labelled fresh or frozen-thawed forages were determined using *in vitro*, *in situ* and *in vivo* methods. The first two methods were used for reasons of simplicity, repeatability and cost effectiveness but, as shown later, there are some differences between results obtained by these methods and those obtained *in vivo* in sheep ingesting the same forages. It seemed therefore, that only *in vivo* experiments can provide certainty about the rate and extent of degradation of fresh lucerne and ryegrass in the rumen. Accordingly, *in vivo* experiment was conducted in which sheep ingested ^{15}N-labelled ryegrass, and the flows of ^{15}N through peptide, amino acid, ammonia and microbial N pools were determined. The *in vivo* work was done with ^{15}N-labelled lucerne but the results are not presented in

this thesis because of submission deadline. The results obtained with ryegrass *in vivo* were compared with those obtained using *in vitro* and rumen *in situ* methods.

Chapter 2 Metabolism of nitrogen of fresh or preserved forages in the rumen

2.1 Introduction

This chapter contains a review of nitrogen (N) sources and metabolism in the rumen. Proteolytic enzyme sources and mechanisms of degradation of dietary and microbial protein are also reviewed. Methods of estimating microbial protein synthesis (MPS), gas production techniques, using digesta flow markers and of ^{15}N-labelled feeds are also included. The importance of, and basic physiology of two plants species, lucerne *(Medicago sativa L.)* and perennial ryegrass (*Lolium perenne* L.) as sources of protein for ruminants are also reviewed.

2.2 Fresh forage

Ruminant livestock in Australia are generally dependent on grazed pasture. The pasture can be grazed directly or harvested and stored as hay or silage for use during feed shortage. Generally, pastures contain mixtures of plants including grasses such as perennial ryegrass and kikuyu, and legumes such as lucerne or white clover. The nutritive value of the material available also depends on the stage of maturity of the plant at the time of grazing or harvesting, and on interactions between the pasture and the animals grazing them. In general, grasses provide the benefits of

high DM yields and legumes provide material with high protein concentrations. The use of fresh forages is the cheapest way of providing feed to ruminant livestock and, in Australia, animals can graze continuously throughout the year (Moran 1996). Furthermore, animal products from grazing animals have healthy attributes. For instance, milk from cows grazing fresh forage is higher in conjugated linoleic acid than milk from cows on preserved forage (AbuGhazaleh *et al.* 2003; Schroeder *et al.* 2003)

Fresh forages also have disadvantages. Compared to dried forage, when the herbage initially contains less than 25% DM, voluntary intake of fresh forage decreases with its water content. The resulting DMI decreases by 1.8-3.2 g/kg$W^{0.75}$ for each 10 g/kg increase in water content (Grant *et al.* 1974; John and Ulyatt 1987). On the other hand, nutritional and production research with fresh forage has been faced with the difficulty of measuring or estimating feed intake by the animal while grazing (Thomson 1982). Fresh forages may also contain hazardous pathogens or toxins which potentially damage animal health and hence product quality (Hinton 2000). In studies of the digestion of fresh forages, it is necessary to have both animals and forages ready at the same time. Any long-term delay may cause forages to mature and their nutritive values will change.

2.2.1 Effects of plant anatomy on intake and digestibility

Forages commonly contain two main components, viz. cell contents and cell wall. The cell contents contain the majority of organic acids, soluble carbohydrates, CP, fats, and soluble ash while the cell-wall fraction includes hemicellulose, cellulose, lignin, cutin, and silica (van Soest 1982). Minson (1990) used microscopy to view forage material and found that plants have five diverse types of tissues. These were the vascular bundles containing phloem and xylem cells, the parenchyma bundle sheath surrounding the vascular tissue, sclerenchyma patches linking the vascular bundles to the epidermis, mesophyll cells among the vascular bundles and epidermal layers, and a single external layer of epidermal cells enclosed by a protective cuticle. Minson (1990) also developed a simple model that linked plant

anatomy and chemical composition which was helpful for comparing differences in potential digestibility between different fractions (See Figure 2-1). In this model, different cell types resistancy to rumen digestion are as follows: phloem and mesophyll < epidermis and parenchyma bundle sheath < sclerenchyma < lignified vascular tissue. However, other factors such as species, leaf *v.* stem, growing phase and management are also important and affect plant digestibility in the rumen.

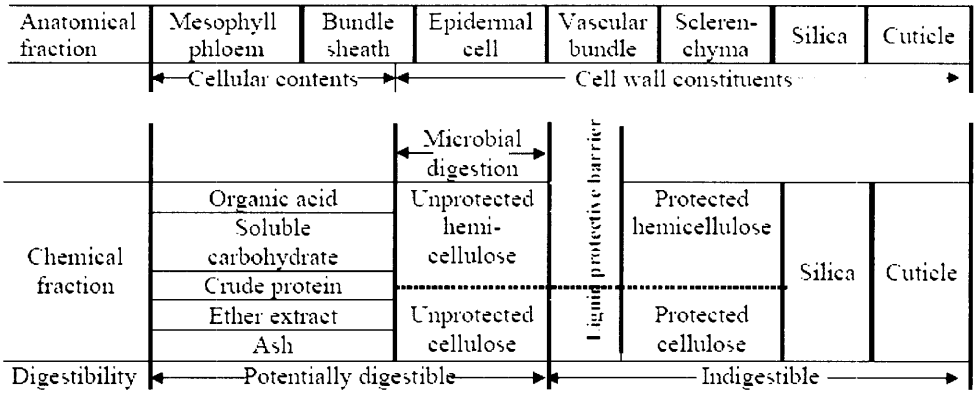


Figure 2-1. A theoretical model of plant anatomy and chemical fractions (adapted from Minson 1990)

2.2.2 Plant maturity

Albrecht *et al.* (1987) found there was a negative relationship between physiological maturity of forages and the ratio of leaf to stem. They also found that lucerne stems increased in cell wall and lignin concentrations with advancing maturity while lucerne leaves remained relatively constant. Thus, lucerne stem digestibility decreased considerably with increasing maturity whereas the digestibility of lucerne leaves was little affected. Digestion kinetics of five mature grass species (green and non-senescent) were investigated by Chaves *et al.* (2006) using rumen *in situ* and *in vitro* incubations. Using the *in situ* method, these workers showed there was a slow colonisation of all components, but especially stem, which limited the rate of nutrient release. Also, effective degradability was highest for leaf

but speed and extent of particle size reduction by chewing during eating and ruminating affected the degradation rates *in vivo*.

As grasses mature, highly digestible leaves form a smaller fraction of the plant while the less digestible stems become more lignified, so the overall rumen digestibility decreases. Legumes maintain higher leaf to stem ratio with advancing maturity and their leaves are more digestible than in grass, although the effects of maturity on feed quality differs with different types of legumes (Moran 1996).

At the same stage of maturity grasses usually contain higher cell wall but lower lignin concentrations than legumes (Van Soest 1994). Ruminants generally ingest temperate, but not tropical, legumes in greater quantities than grasses (Minson 1990). The higher digestibility of legumes is associated with their proportionally lower cell wall contents and lower resistance to breakdown during eating and rumination. Thornton and Minson (1972) found that, at the same digestibility (about 50%), the voluntary intake of sheep was 28% higher and ruminal retention time of digesta was 17% shorter for legumes than for grasses.

Smith *et al.* (1972) found that the lignin to cellulose ratio in the indigestible residues of grasses and legumes was similar (0.94 v. 1.09, respectively) and suggested that, in both forage species, almost the same amount of cell wall polysaccharides were protected from digestion by lignin. Dewayne (1993) pointed out that intake of legumes, compared to grass, is higher because they have less of a cell wall and lower resistance to ruminal degradation.Wilson and Kennedy (1996) also suggested that the higher digestibility of legumes compared with grasses might be related to leaf length. In their review, Oba and Allen (1999) found greater NDF digestibility for grasses than legumes due to the fact that NDF filling effect in the rumen might be less for legumes because of greater particle fragility and shorter rumen retention time compared with grass. Grass particles are naturally long, with a low functional specific gravity, and are easily entangled in the rumen. Chewed vascular particles of legumes are shorter and thicker and have a high functional specific gravity; they are therefore likely to escape more quickly from the rumen. Thus, potential intake of plant material is dependent not only on its fibre content, but

also on the anatomy of the plant and the way in which it breaks down during digestion.

2.2.3 Degradation of fresh forage in the rumen

The degradation of plant protein in the rumen is normally catalysed by enzymes of rumen micro-organisms including bacteria, fungi and protozoa; however, when animals ingest fresh forages, plant enzymes can contribute significantly to proteolytic activity (Theodorou *et al.* 1996). Recent studies have shown that plant enzymes contribute to degradation of protein in fresh forages soon after they arrive in the rumen. Plant enzymes have different functions that include the dissolution of storage proteins during seed germination, the promotion of protein turnover, and degradation of damaged proteins (Hrmova and Fincher 2001). The activity of endogenous peptidases and their molecular weight and class in a subsample in grass and legume forages was investigated in an *in vitro* experiment by (Pichard *et al.* 2006). They indicated that there was a wide range (from 0.00 to 1.30) in proteolytic activity index (PAI) and it was due to differences in genus, species, and cultivar. The wide variation in proteolysis observed and the predominant occurrence of one peptidase of the same enzyme class support the idea that a reduction of protease activity in forages can be achieved by genetic improvement.

Fresh herbages, including grasses and legumes, contain 15-30% CP and 6-22% of rumen fermentable carbohydrates (Ulyatt 1971). Fresh forages showed high apparent digestibility of OM and total N when ingested by both sheep and cattle (Beever *et al.* 1986; MacRae and Ulyatt 1974). For cattle grazing on either perennial ryegrass or white clover, Beever *et al.* (1986) estimated that about 25% of duodenal NAN flowing to the small intestine was undegraded dietary N and about 72% was microbial N. They also found that when forage N content was greater than 25-30 g N/kg DM, there was rapid fermentation of fresh forage N to ammonia and excess ammonia was absorbed from the rumen.

Archimede *et al* .(1999) reported that DM intake of sheep, and total tract digestibility of OM, CP, NDF and ADF were higher when they consumed fresh

rather than dried *Digitaria decumbens*. They also found that urinary N excretion was slightly lower with dried rather than fresh forage, and microbial N flow was also higher in animals ingesting fresh material, even though the efficiency of microbial synthesis and total N duodenal flow was similar for both forms.

The rate of degradation of fresh forage in the rumen is also affected by the stage of harvest (stage of maturity of the plant when it is ingested). Plant maturity leads to an important decrease of rumen use of the whole forage, but it has little effect on rumen degradation of CP (Gonzalez *et al.* 2001). Sanderson and Wedin (1989) found that concentrations of N in ADF in leaves, stems, and total herbage of lucerne and clover were twice that of grass ADF. This may be due to greater lignin concentrations generally reported in legumes. Lucerne has a higher rate of rumen DM degradation than grasses; however, the extent of CP degradation does not differ among these two forages (Elizalde *et al.* 1999). Aufrere *et al.* (2002) found that the effective degradability of N for fresh red clover, estimated by the rumen *in situ* method, did not vary with the stage of growth and the concentration of total N and NAN in rumen fluid were low.

2.3 Nitrogen sources for ruminants

2.3.1 Ingested feed

Most of the N available for digestion and metabolism in the rumen is supplied by the ingested feedstuffs. Forage N is present in different forms that can be divided to protein and non-protein nitrogen (NPN). In all forage species, the protein content decreases as the plant matures. After harvest, processing such as making silage also affects the forage protein content. Silages have a higher NPN concentration than fresh forages because of the fermentation of plant protein by plant and microbial enzymes during the ensiling process (McDonald 1982). The NPN fraction consists of free amino acids, nucleic acids (DNA and RNA) ,nitrate, short chain peptides, ammonia and non volatile amines (Givens *et al.* 2000a; Mangan 1982). The NAN degrades rapidly in the rumen and provides a N substrate for microbial-N synthesis almost immediately.

In fresh forage, most of the proteins are present as leaf proteins. Approximately 75% of the leaf protein is found in the chloroplasts of which half is Fraction 1 (18S) protein, consisting of ribulose-1,5 biphosphate carboxylase oxygenase (RUBISCO) (Makoni 1993). This protein contributes a large proportion of the protein available for metabolism in the rumen of animals ingesting fresh forage as it is readily released from the plant tissue during mastication (Mangan 1982). Nugent *et al.* (1983) found that about 30 – 40% of the total soluble protein in lucerne is present as Fraction 1. They also demonstrated that Fraction 1 protein is highly degradable in the rumen suggesting that few of the amino acids from this protein would escape undegraded from the rumen and become directly available for the host animal. However, Min *et al* .(2000) indicated that the ruminal degradation of Fraction 1 protein molecule is not uniform: the small sub-unit of the protein (MW 16 KD) is degraded at a slower rate than the large sub-unit (MW 56 KD).

Fractions 2 and 3 represent smaller proportions of leaf proteins. The Fraction 2 protein represents about 25% of the total leaf protein and contains a mixture of cytoplasmic and chloroplastic soluble proteins. The chloroplast membrane protein (Fraction 3) is insoluble and comprises about 40% of the chloroplast protein. Mangan (1982) proposed that some of these Fraction 2 and Fraction 3 proteins escape rumen fermentation.

2.3.2 Endogenous N

Recycled urea can be a major source of N for rumen micro-organisms (Sarraseca *et al.* 1998). The amount of urea recycled via saliva is directly related to blood urea concentration and flow rate of saliva (Nolan and Leng 1972) as well as blood urea-N (BUN) concentration, rumen ammonia concentration and the fermentable substrate availability in the rumen (Kennedy and Milligan 1978). The contribution of recycled urea to the rumen ammonia pool when urea is hydrolyzed by microbial urease is higher when rumen ammonia concentration is relatively low because of the low level of N in diet and low feed N degradability. Nolan and Stachiw (1979) indicated in sheep fed low quality roughage, about half of the total N in rumen ammonia was derived from endogenous urea. The amount of saliva

secreted can be greatly influenced by feed intake and the physical structure of the diet (Kay 1966). Blood urea nitrogen (BUN) concentration is influenced by the extent to which absorbed amino acids (AA) are oxidized and by the rate of absorption of ammonia from the rumen. Therefore, BUN concentration reflects N balance in the diet (Neutze *et al.* 1986). Saliva also contains a mixture of proteins (Somers 1961). Rumen epithelium is continuously sloughed into the rumen providing a potential N source for the rumen micro-organisms (Orskov *et al.* 1980) and urea is also transferred through the rumen wall. Together, endogenous urea and non-urea N can be important sources of N for microbial growth in the rumen of animals on low protein diets. Unfortunately, factors affecting the entry of endogenous proteins into the rumen and their ruminal metabolism or outflow from the rumen are still not clearly understood. Endogenous proteins also enter the gut post-ruminally (Hart and Leibholz 1990) but the rates of protein entry have proved to be difficult to determine in this part of the gut (for review see Lapierre and Lobley 2001).

2.4 Proteolytic enzymes sources in the rumen

2.4.1 Rumen microbes

Rumen bacteria are responsible for much of the catabolism of protein in the rumen (Brock *et al.* 1982; Nugent and Mangan 1981). Most proteases and peptidases in ruminal contents are closely associated with the outer layer of the bacterial cell wall, and the enzymes can be easily liberated by using detergents (Brock *et al.* 1982; Kopecny and Wallace 1982; Prins and Clarke 1980). About 30-50% of the rumen bacteria species isolated from rumen fluid have been shown to be proteolytic (Bryant and Burkey 1953; Fulghum and Moore 1963; Prins *et al.* 1983). Brock *et al.* (1982) found that both rumen bacteria and protozoa exhibited proteolytic activity, but the bacteria had a specific activity that was 6-10 times higher than that of protozoa. Bacterial proteases are the main enzymes catalyzing breakdown of soluble proteins such as casein, azocasein and Fraction 1 leaf proteins (Nugent *et al.* 1983). The characteristics and proteolytic activity of each rumen bacterial species has been reviewed and *Bacteriodes succinogenes, Ruminococcus flavefacines, Ruminococcus*

albus and *Bacteroides amylophylus* are reported to be the most important proteolytic rumen bacteria present in the rumen in different feeding systems (Hobson 1988).

The rumen often contains a large (up to 10^6/ml) and varied population of ciliated protozoa which, at times may represent over 50% of the total microbial biomass (Walker *et al.* 2005; Williams 1992). Protozoa are responsible for some of the degradation of insoluble particulate protein but have a limited ability to assimilate peptides or AA (Bird *et al.* 1990). The mechanisms of consumption and degradation of bacteria by protozoa is well documented by Hristov and Jouany (2005) and it is possibly the most important factor influencing bacterial lysis in the rumen (Wallace and McPherson, 1987). Ruminal ciliates are composed of two orders, *Entodiniomorphida* and *Holotrichs*. *Entodiniomorphida* protozoa are particularly efficient in taking up particulate matter suspended in ruminal fluid (Jouany 1996). Michalowski (1975) tested the effect of different proteins on the growth of *Entodiniomorphida* protozoa cultivated *in vitro* and found that these ciliates do not assimilate or metabolize soluble proteins. *Holotrichs* show multiple forms of protease but the actual contribution of individual genera to protein degradation is unclear (Williams 1992).

2.4.2 Plant material

Plant proteases are activated after harvesting and are responsible for decreasing the plant protein content during wilting (McDonald 1982). Throughout the ensiling process, degradation of plant protein continues under the influence of plant and microbial enzymes during the ensiling process (Merry *et al.* 1999). In this ecosystem, plant enzymes convert plant proteins to lower molecular weight peptides and amino acids which are consequently utilized by the developing silage microflora (McDonald *et al.* 1991; McNabb *et al.* 1994). It has been suggested that plant proteases might remain active in the rumen of animals fed fresh forages (Theodorou *et al.* 1996; Zhu *et al.* 1999a). They also proposed that plant enzymes play an important role in the initial breakdown of plant proteins before they are subject to the action of microbial proteolytic enzymes. In nutrition studies with ruminants, the importance of plant proteases in rumen proteolysis has not been widely considered

because, in most cases, dried and finely ground substrates have been used. With dried or processed feeds, proteolytic enzymes of plant origin could be expected to be denatured so that most of the proteolysis would be due to microbial enzymes (Tolera and Said 1997). Rumen conditions (i.e. temperature of 39°C, lack of light and oxygen and the presence of VFAs) are optimal for many plant proteases (Feller 1995; Vierstra 1996). Although the conditions are stressful for plant cells their proteases are still functional (Theodorou *et al.* 1996). Because of the stress on the plant after it enters the rumen environment, most of the initial and rapid phase of proteolysis in the rumen are caused by plant enzymes (Beha *et al.* 2002; Kingston-Smith and Theodorou 2000; Zhu *et al.* 1999b). Kingston-Smith *et al.* (2003) used incubation media *in vitro* with or without rumen micro-organisms and investigated the extent of membrane damage and release of cell constituents from model and forage. They found that, when plant cells are subjected to rumen conditions, ions start to leak through their cell membranes after 2 h which shows that plant cell membranes were intolerant of rumen conditions.

2.5 Protein degradation mechanisms

Protein concentrations in food for ruminants have been assessed in terms of CP (%N × 6.25) which consists of true protein (TP) and NPN. Rumen microbes are able to degrade both fractions. While proteins and NPN compounds contribute to CP, the nutritive value of CP in feedstuff for ruminants is best explained by the rate and extent of CP degradation in the rumen and the composition of the rumen degradable protein (RDP) and rumen undegradable protein (RUP) fractions. Figure 2-2 summarizes N metabolism in the rumen and animal tissues.

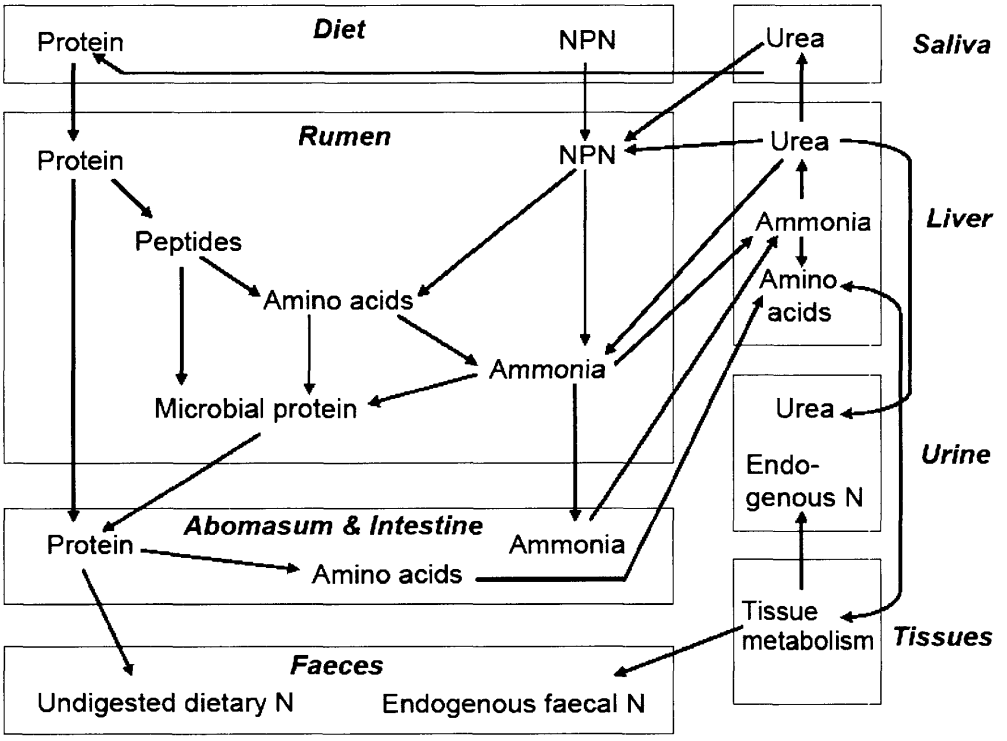


Figure 2-2. Outline of N metabolism in ruminant animals (adapted and modified from Annison and Lewis 1959)

The degradation of protein to AA is a cell-associated extracellular process whereas the degradation of AA to ammonia is an intracellular process (Cotta and Russell 1996). Peptides and AA which are intermediates of protein degradation in the rumen fluid are often present in low concentrations because they are quickly removed from the rumen media by micro-organisms; however, they tend to increase in concentration after feeding (Annison 1956; Broderick and Wallace 1988).

Amino acids in microbial cells are used for microbial protein synthesis or are deaminated to ammonia and keto-acids. Depending on rumen pH and availability of energy and nutrients, ingested feed is fermented by microbes in the rumen and ammonia is produced (Annison *et al.* 1959). Up to 90% of dietary TP and NPN may be degraded to ammonia in the rumen (Volden *et al.* 2002). The ammonia concentration in the rumen fluid ranges from 85 to over 300 mg/l (McDonald *et al.* 1995) but Satter and Slyter (1974) found that the optimum level of ammonia for

efficient microbial cell synthesis *in vitro* was between 50-80 mg/l. Rumen microbes can utilize ammonia as a N source for protein synthesis and, after digestion in the small intestine, the amino acids can be absorbed and used for animal production or meet maintenance requirements (O'Connor *et al.* 1990). Most of the ammonia that is not assimilated by microbes is absorbed across the rumen wall and passes into the portal blood and travels to the liver. Some researchers have measured the transfer of ammonia through the rumen wall using intraruminally administered ^{15}N-labelled ammonium salts. There was a linear relationship between ammonia absorption rate and ammonium concentration in rumen fluid (Obara *et al.* 1991; Siddons *et al.* 1985).

If the diet is low in protein, or if the dietary protein is resistant to degradation, rumen ammonia concentration will be low and, if the concentration falls below 50 mg N/L, rumen microbial growth may be restricted (Satter and Slyter 1974). Conversely, if the protein degradation proceeds more rapidly than utilisation of the resulting ammonia, then ammonia will accumulate in rumen contents and exceed the optimum level. In this situation, excess ammonia will be transferred from the blood to the liver and converted to urea. Urea is the main end-product of ammonia and amino-N metabolism in ruminants. There are two ways by which the extra urea in the blood can be removed, viz. excretion via urine or transfer into the gut. Some of the latter is hydrolysed to ammonia in the rumen and may be used by microbes for protein synthesis. Some of this urea may be returned to the rumen via saliva and also directly through the rumen wall, but the greater part may be excreted in urine and hence wasted (McDonald *et al.* 1995).

2.6 Factors affecting protein degradation

Protein degradation is affected by variations in the microbial population in the rumen and by the physical and chemical properties of forages. Peptidases and proteases would have reduced access to the polypeptides when the protein is coated by lipids and water insoluble materials. Effective degradability is a function of the protein degradability and the residence time of the plant protein in the rumen. The factors affecting feed protein degradation have been reviewed (Dewhurst *et al.* 2000;

Klopfenstein *et al.* 2001). It has long been accepted that measuring solubility of protein is correlated with protein degradability (Annison 1956) although this relationship exists, some soluble proteins are slowly degraded (Mahadevan *et al.* 1980). When forages are digested *in situ*, protein is assumed to be degraded according to the following equation proposed by Orskov and McDonald (1979).

$$Deg(t) = a + b[c/(c+k)\ ] \qquad (1)$$

where *Deg(t)* is the fraction of protein degraded at time t of incubation; a = fraction of immediately degradable (soluble) protein; b = insoluble but degradable protein; c = the rate constant for degradation of fraction b (h^{-1}).

Few studies of protein degradability have been carried out using fresh forages. Higher solubility materials are more rapidly degraded in the rumen (Beever and Siddons 1986). Temperate legumes have condensed tannins which make an insoluble compound with proteins and decrease N solubility. High concentrations of condensed tannins (50-100 g/kg DM) are toxic for rumen micro-organisms, but lower concentrations reduce the NPN solubility of silage and improve the efficiency of protein utilization (Albrecht and Muck 1991; McNabb *et al.* 1996).

Protein structure can affect the rate of protein degradation. Hydrolysis of protein depends on the accessibility of the hydrolysable sites in the polypeptide chain. This accessibility depends on the types of enzymes involved, and on conditions at the binding site (i.e. pH, availability of cofactors). The extent of secondary and tertiary structure and presence of disulfide (S-S) bridges that link two cysteine residues in different parts of the same polypeptide chain or in different polypeptide chains both result in lower protein degradation rates (Mahadevan *et al.* 1980). Chemicals such as mercaptoethanol or performic acid increase the rate of proteolysis of soluble proteins with disulfide bridges (Means and Feeney 1971). However, some soluble proteins (*e.g.* casein and Fraction 1 protein) without disulfide bridges proteolysis were not affected (Nugent *et al.* 1983). Degradability of some of proteins such as zein and casein are related to solubility but soluble albumins are slowly degraded. So, it can be concluded that both solubility and structure can affect protein degradability (Annison 1956).

Soluble proteins encourage proteolytic microbes in the rumen. Nugent and Mangan (1981) indicated that the degradation rate of Fraction 1 protein in fresh lucerne is 9 times greater than with hay-fed animals supplemented with concentrate. Hazlewood *et al.* (1983) concluded that when hay was replaced by fresh forage, rich in soluble protein, ruminal proteolytic activity was increased and when bacteria were given Fraction 1 protein as their only N source, degradation rate was increased significantly.

2.7 Methods for determination of degradation of dietary protein

The characteristics of dietary protein degradation have been determined by *in vitro*, rumen *in situ* and *in vivo* methods. The choice of method depends on the research objectives and can affect the mathematical model used and interpretation of results. There have been some useful reviews about application of these methods (Kitessa *et al.* 1999; Schwab *et al.* 2003; Vanzant *et al.* 1996).

2.7.1 *In vivo*

I*n vivo* procedures are used to measure the flow of total and microbial proteins to the duodenum or abomasum. Generally, animals are fitted with a cannula in the abomasum or duodenum and non-absorbable gut markers are used (Faichney 1975) to enable the flow of digesta past these cannulas to be determined. It is then necessary to identify the total N, microbial N and endogenous N flowing within digesta so that undegraded feed N can be determined by difference. Since they more nearly represent the real response of animals to experimental conditions, *in vivo* techniques can be used as a reference to evaluate simpler methods; However, *in vivo* results will depend on the experimental conditions when measurements are carried out (Givens *et al.* 2000b).

In vivo estimation of protein degradability in the rumen requires separation of the protein flowing to the duodenum into undegraded feed, microbial and

endogenous protein. Total protein and the proportion of microbial protein entering the duodenum are determined by using a suitable microbial marker. Broderick and Merchen (1992) reviewed the use of markers for quantifying microbial protein synthesis in the rumen and concluded that the most reliable results can be obtained when the ^{15}N method and total purine determination are used together. However, in their review, Reynal *et al.* (2005) concluded that theoretically, the external marker ^{15}N offers several advantages over total purines (TP). The ^{15}N should be evenly distributed throughout the microbial cell; thus, microbial lysis during isolation, although not affecting ^{15}N enrichment, would result in loss of purines from cell cytoplasm, lowering the purine:N ratio and leading to overestimation of microbial flows (Martin-Orué et al., 1998; Carro and Miller, 2002). Moreover, ^{15}N-labeled protein leaving the rumen that was enriched in excess of natural abundance will only be of microbial origin, whereas a portion of the purines leaving the rumen may be of dietary origin (Pérez et al., 1996b; Vicente et al., 2004). However, results from comparisons between TP and ^{15}N reported in the literature have been inconsistent. The use of TP resulted in higher (Carro and Miller, 2002), lower (Firkins et al., 1987b; Pérez et al., 1996a), or similar (Calsamiglia et al., 1996; Pérez et al., 1997a) estimates of microbial yield compared with ^{15}N.

Ouellet *et al.* (2002) estimated that endogenous N flows at the duodenum and found that endogenous secretions into the small intestine may contribute 30% towards total protein turnover across the digestive tract and, therefore, represent a major contribution to both the N and energy economy of lactating animals. The *in vivo* measurements of ruminal protein degradation are expensive, time consuming, labor intensive, and subject to error due to inaccurate estimation of endogenous proteins as well as to uncertainty associated with differentiation of feed and microbial proteins using markers (Stern *et al.* 1994).

Hristov (2007) compared sampling reticular content as an alternative to duodenal sampling for estimation of nutrients and microbial protein outflow from the rumen. A third method based on sampling of ruminal contents was also tested. Reticular digesta had lower OM and greater NDF contents than the duodenum digesta and the proportion of microbial N was particularly greater in the fluid phase

of reticular digesta. Microbial N flow estimated based on ruminal sampling was similar to the amount estimated by both other methods. He also found that ruminal digestibility of OM, N, fiber fractions and microbial protein outflow from the rumen can be measured based on either sampling of ruminal or reticular digesta.

2.7.2 *In vitro*

In vitro methods not only have the advantage of being less expensive and less time consuming, but experimental conditions can be more completely controlled than with *in vivo* experiments. On the other hand, application of *in vitro* results to the prediction of *in vivo* events is dependent on how well the conditions are known for the *in vivo* event in question (Raab *et al.* 1983). Dietary protein degradation in the rumen is frequently estimated *in vitro* by incubating feeds with a wide range of commercial proteases. Some researchers have argued that the actions of these kinds of proteases are not the same as microbial enzymes. Luchini *et al.* (1996) concluded that the degradation rate of dietary protein was lower with commercial proteases than with proteases from rumen fluid. Because commercial proteases did not mimic the degradation rates obtained with strained rumen fluid, commercial enzymes will prove unreliable for estimating rate and extent of ruminal protein degradation. Ammonia production during incubation of feed with rumen fluid *in vitro* can be used as an indicator of the rate and extent of protein degradation (Little *et al.* 1963). However, ammonia is assimilated by microbes while it is being produced. The enrichment ratio of microbes incubated with ^{15}N-ammonia can be used to indicate the utilisation of ammonia-N by microbes. Hristov and Broderick (1994) estimated rate and extent of ruminal protein degradation using $^{15}NH_3$ to quantify uptake of protein degradation products for microbial protein synthesis *in vitro*. However, they could not find useful data for ruminal protein degradability from ammonia because because direct microbial incorporation of non-NH3 N differed among proteins with different degradation rates. However, they concluded that reasonable estimates of rate and extent of protein degradation for seven standard proteins was achieved when net release of NH_3-N plus net formation of microbial N, calculated by subtracting blank concentrations.

An inhibitor *in vitro* (**IIV**) method for estimating protein degradation rate, from net appearance of N as NH_3 and TAA in ruminal media containing 1.0 m*M* HS and 30 mg/L of CAP, was developed by (Broderick 1978; 1987). With this approach, substrate-limiting amounts of protein (i.e., under first-order conditions) were incubated with ruminal inocula containing metabolic inhibitors to obtain quantitative recovery of breakdown products. Degradation rate was derived from the time-course of N appearance as total free AA (**TAA**) plus NH_3. In their experiments (Broderick *et al.* 2004) concluded adding NH_3 plus free and peptide-bound amino acids to the inoculum reduced apparent degradation rates, possibly via end-product inhibition.

2.7.3 Rumen *in Situ* method

The suspension of feed materials into the rumen (e.g., *in situ* technique, artificial fiber bag technique) allows intimate contact of the test feed with the ruminal environment. Rumen environment (temperature, pH, buffer substrate and enzymes) is the same when tested feed is incubated in nylon bag, although in the ruminal environment, the feed is not subjected to the total ruminal experience: i.e., mastication, rumination, and passage. With the development of new protein evaluation systems, the *in situ* method has commonly been used as a reference method for validation of other laboratory methods. With the *in situ* method, small feed samples are incubated in porous nylon or polyester bags placed in the rumen of an animal on a diet that contains ingredients similar to those being evaluated in the bag. The size of the pores in bags is critical and should always be reported. The pores should be small enough to retain the feed sample, but large enough to allow bacteria to enter the bags. Because just small amounts of feed sample are incubated, the overall rumen fermentation in the rumen itself will not be affected, and it is assumed that the conditions within the bags are similar to the conditions in the surrounding rumen contents (Givens *et al.* 2000b). Quin (1938), cited by Givens *et al* .(2000b), was apparently the first researcher to use the technique. He incubated small feed samples in porous silk bags in the rumen to estimate fermentation in the rumen of sheep. Orskov and McDonald (1979) adjusted the prediction of degradability by allowing for the residence time of the digesta in the rumen (fractional rumen outflow

rate), thereby transforming the degradability values to effective degradability (ED). The *in situ* method has been thoroughly reviewed by Huntington and Givens (1995). They recommended that sample preparation before rumen incubation (particularly for low DM feeds) and post-rumen incubation processing of bags and their residues should be standardized. Other aspects requiring standardization include type of cloth, pore size of the bag, bag mobility with respect to cord length and anchor weight, sequence of bag incubation and ratio of sample particle size to bag pore size.

2.8 Nitrogen utilization in the rumen

2.8.1 Peptides and amino acids

Free amino acids and peptides are degraded rapidly by rumen micro-organisms (Wright and Hungate 1967). The accumulation and pattern of flux of these intermediates of protein degradation depends on the type of dietary protein present. Rapidly degraded proteins like casein would be expected to be hydrolyzed faster than the resulting peptides and amino acids (Wallace 1992). Thus, hydrolysis of casein and of similar proteins would be more likely to result in accumulation of peptides and amino acids in rumen fluid. In contrast, a slowly degradable protein like albumin would lead to less accumulation of these intermediates.

The mechanisms involved in the accumulation of peptides during ruminal protein degradation are being investigated. Figure 2-3 illustrates a scheme that has been proposed to explain the utilization of proteins by ruminal bacteria (Russell *et al.* 1991). According to this scheme, the protein utilization by ruminal micro-organisms is a multi-step process involving proteolysis, peptide hydrolysis, the uptake of peptides or amino acids into the microbial cells and their use for protein synthesis or as a source of energy for the cell. As the protein utilization by ruminal micro-organisms occurs in several steps that yield several intermediate products with different degradation characteristics, accumulation of these intermediates during dietary protein degradation is possible. Proteolysis of dietary proteins results in the production of a variety of peptides in the rumen.

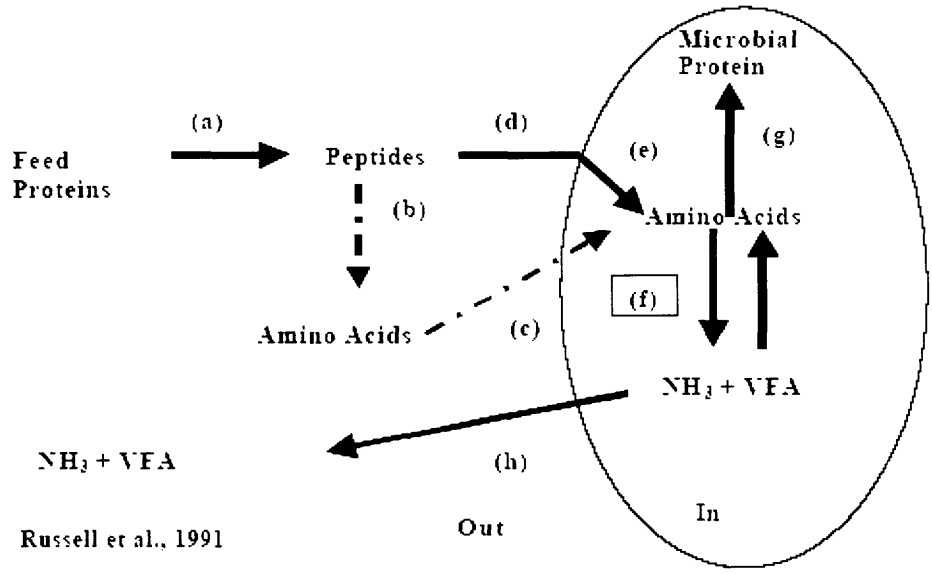


Figure 2-3. A schematic representation of the protein utilization by ruminal bacteria showing (a) proteolysis, (b) extracellular peptide hydrolysis, (c) amino acid transport, (d) peptide transport, (e) intracellular peptide hydrolysis, (f) amino acid fermentation, (g) microbial protein synthesis and (h) diffusion of ammonia and VFA. (adapted from Russell *et al.* 1991)

Most studies of ruminal metabolism of peptides and amino acids by microbes have been done *in vitro*. Peptides are utilized more rapidly by mixed rumen micro-organisms and incorporated into cellular materials more efficiently than free amino acids (Prins 1977). The reason for the higher efficiency is not clear, but Payne (1983) indicated that, at least in *E. coli,* it might be energetically more efficient for the cells to convey peptides rather than amino acids into bacterial cells. Small peptides are converted to VFA, while a higher proportion of larger molecules tend to be incorporated into bacterial protein (Wright and Hungate 1967).

Soto *et al.* (1994) found that bacterial growth rates were stimulated by more than 70% in the presence of peptides rather than amino acids. They concluded that fermentation by rumen bacteria is not limited by the availability of peptides or amino acids when ammonia is available, and that growth rate is limited by a slowly degradable energy source. Russell *et al.* (1983) found that, for maximal microbial

protein synthesis, non-structural carbohydrate (NSC) fermenting bacteria obtained 67% of their N from peptides or amino acids. Other workers have found that there is a linear relationship between RDP, ruminal peptide and ammonia-N concentration (Fu *et al.* 2001).

The influence of peptides and amino acids on fermentation rate and *de novo* synthesis of amino acids by rumen micro-organisms was investigated by Atasoglu *et al.* (1999). They concluded that when the concentration of peptides decreased, the proportion of microbial-N formed from NH_3 increased. They also concluded that different individual amino acids are synthesized *de novo* to different extents by rumen micro-organisms when pre-formed amino acids are present.

2.8.2 Ammonia concentration and microbial assimilation

The ammonia concentration in rumen fluid at any time is a balance between its production rate (via amino acids deamination, epithelial sloughed cells, degradation of endogenous urea and secretion by protozoa) and its uptake rate (via microbial utilisation, absorption through the rumen wall and outflow to the lower digestive tract).

Ammonia concentration in rumen fluid is determined by the rate of production of ammonia as a result of the metabolism of protein and NPN in dietary and endogenous materials and the rate of removal of ammonia by microbial assimilation and by passage through the rumen wall. Passage of ammonia through the rumen wall depends on pH which determines the ratio of ionized (NH_4^+) to un-ionized (NH_3) ammonia species in rumen fluid. The transport of ammonia occurs in two forms: in the lipophilic form as NH_3, the magnitude of which is linearly related to the pH in the ruminal fluid at pH values above 7, while at a physiological pH of 6.5 or lower, ammonia is predominantly absorbed as NH_4^+ via putative potassium channels in the apical membrane (Abdoun *et al.* 2006). NH_3 is more readily absorbed than NH_4^+ and ammonia assimilation by micro-organisms is favoured by higher concentrations of NH_3 (Hogan and Weston 1969; Nolan *et al.* 2007).

a) Production

The effects of ammonia concentration and protein solubility on the rate and extent of protein degradation by total mixed rumen micro-organisms or mixed rumen bacteria was investigated by Song and Kennelly (1991). They used ^{14}C-labelled soybean meal and concluded that proteolytic activity of rumen microbes as well as ammonia concentration depends on solubility and protein structure. If dietary protein is highly degradable, production of ammonia will exceed the microbial requirements (see Section 2.5). ,

b) Assimilation

The mechanisms of ammonia assimilation by ruminal microbes have been described by Wallace *et al.* (1997). Some cellulolytic bacterial species such as *Fibrobacter succinogenes, Ruminococcus flavefacines* and *Ruminococcus albus* can utilize only ammonia, and about 89% of the species tested by Allison (1969) could use ammonia as well as other forms of N. Using ^{15}N-ammonia, Atasoglu and Wallace (2002) investigated the effect of ammonia concentration on its rate of incorporation and use for *de novo* synthesis of amino acids by three main non-cellulolytic species of ruminal bacteria, *Prevotella bryantii B14, Selenomonas ruminantium HD4* and *Streptococcus bovis ES1.* Their results showed that ammonia concentration has an important effect on *de novo* synthesis of bacterial protein in the non-cellulolytic ruminal bacteria and this effect depends on bacterial species.

In vivo studies using ^{15}N-ammonia have also shown that, the percentage of microbial-N synthesized from ammonia-N may vary between 40 and 90% in animals given different diets (Nolan *et al.* 1972; Nolan and Stachiw 1979; Vanderwalt 1993). When the diet contains true protein and there is a rapid rate of release of peptides and amino acids, more bacterial-N is derived from peptides and amino acids and less is synthesized from ammonia (Salter *et al.* 1979). Hristov (1997) designed an experiment to investigate the effect of different levels of carbohydrates and simultaneous provision of ammonia and amino N on utilization of α-amino N by

mixed ruminal microorganisms. Their finding indicated that bacterial preference to ammonia or non-ammonia N depends on the availability of energy and ammonia-N.

There are two enzymes involved for microbial ammonia assimilation, *viz.* glutamate dehydrogenase and glutamine synthetase which can convert ammonia to amide-N, after which it can be assimilated into the amino group of intracellular free amino acids (Salter *et al.* 1979). These enzymes incorporate ammonia into the amides, glutamine and asparagine, that are later used to aminate α–ketoglutarate to form glutamate (Erfle *et al.* 1977). Alanine and aspartate are synthesized from glutamate and these are in turn used to form other amino acids. Ammonia assimilation occurs principally by the action of NAD- and NADP-linked glutamate dehydrogenases. The ATP-dependent glutamine synthetase-glutamate synthase (GS-GOGAT) couple, which traps ammonia in glutamine has low activity in normal rumen ammonia concentrations but is active when rumen ammonia concentration is low. However, GS-GOGAT might be important for bacterial survival, particularly where ammonia concentration is limiting (Walker *et al.* 2005).

Blake *et al.* (1983) carried out an experiment *in vivo* to investigate the pathways of ammonia incorporation into free amino acids within the bacteria. They infused a solution of $^{15}NH_4Cl$ into the rumen and found that the protein-free cell supernatant fraction was rapidly labelled whereas bacterial protein and cell wall fractions were labelled quite slowly. They also found that the levels of enrichment of the amino acids, alanine and glutamate, that occurred in the protein-free cell supernatant fraction suggested that the enzymes, alanine dehydrogenase and glutamate dehydrogenase are the major enzymes for assimilating ammonia when concentrations of soluble carbohydrate and ammonia are high in the rumen.

Satter and Slyter (1974) suggested that a minimum concentration of 50 mg/l ammonia-N is required for optimal microbial growth in the rumen. At lower concentrations (less than 10-20 mg/l NH_3-N), efficiency of ammonia utilization is higher because almost all of the ruminal ammonia is used by the microbial population and so loss of ammonia by absorption from the gut is minimal; however, as ruminal ammonia begins to accumulate, the efficiency of utilization of ammonia-N decreases as more is absorbed (Roffler and Satter 1975).

2.9 Microbial protein synthesis in the rumen

The microbial biomass consists of microbes from different species including ruminal bacteria, protozoa and fungi (Hungate 1966). The microbial cells pass to the small intestine and may contribute 40 to 90% of the total amino acids entering the small intestine. Microbial growth rate depends on the availability of different materials such as fermentable carbohydrates, nitrogenous sources, lipids, nucleic acids, minerals and vitamins. However, under normal feeding conditions, energy is the only factor limitng microbial synthesis (Stern and Hoover 1979).

ATP is formed by the fermentation of feed OM and is used by microbes for maintenance and growth purposes. Microbial growth can be defined as molar growth yield (Y_{ATP} = g cell DM per mole ATP). Factors which affect the efficiency of net microbial outflow from the rumen have been reviewed by Dewhurst *et al.* (2000) and include factors such as quality of diet, feed intake, feed particle retention time and synchronization of energy and protein.

Protozoa rely on bacteria as their major source of nitrogenous materials. Bacterial proteins are degraded within protozoal cells to small peptides then to free amino acids that are used for protozoal protein synthesis (Coleman 1972). Ingestion of bacteria and their subsequent lysis by protozoa provides them with essential amino acids. However, some species of protozoa use plant protein and free amino acids (Hobson 1988). Release of digestion products by ciliates is an important part of N recycling in the rumen because the bacteria can utilize the amino acids released from the lyzed protozoa for growth. Viable protozoa metabolize protein and release ammonia as an end-product of their amino acid metabolism.

Hsu *et al.* (1991a) found defaunation decreased ruminal ammonia concentration and CP flow to the duodenum increased due an increase in both bacterial and non-bacterial CP flows. However, Jouany (1991) reported degradation of plant cell wall materials decreased when protozoa were eliminated from the rumen. These workers also suggested that defaunation was associated with an increase in ruminal volume. Retention time of plant particles would likely increase in

defaunated animals because fractional outflow rate of ruminal contents generally is inversely related to ruminal volume (Jouany 1996). A decrease in the duodenal flow of bacterial biomass was observed after inoculation of protozoa into defaunated rumens, probably reflecting an increase in bacterial predation and competition for dietary nutrients from protozoa (Bird and Leng 1978).

Ivan *et al* (2000) found that ruminal presence of total fauna or Entodinium decreased the nonammonia N by 16 and 17%, and total AA flows from the stomach by 20 and 19%, respectively. Flow of bacteria N was decreased in wethers fed the two diets when Entodinium or total fauna were present in the rumen. The presence of Isotricha resulted in higher flow of bacteria N in wethers fed the haycrop diet, but the presence of Dasytricha resulted in higher bacteria N flow in wethers fed the corn silage diet. Entodinium was the most detrimental of ciliate protozoa species concerning protein nutrition of the host ruminant.

As a consequence by increasing dietary and microbial protein flow to the duodenum of ruminants, the intestinal supply of non-ammonia N is always significantly improved by defaunation (Kayouli *et al.* 1986).

2.10 Estimation of microbial protein synthesis

Proteins entering the small intestine are a combination of dietary, microbial and endogenous sources. Estimating the ruminal microbial protein flow is essential to quantifying ruminal escape of dietary protein and microbial yields and various methods have been developed to measure these flows. With these methods, digesta flow to the duodenum or abomasum is estimated and microbial fractions are determined using internal markers such as purine derivatives (PD), DAPA (diaminopimelic acid), RNA (ribonucleic acid) and external markers (e.g., ^{15}N, ^{35}S). The ideal marker should not be present in feed materials; it should also be stable biologically under specific experimental conditions and be present as a constant percentage of the microbial cells in all stages of growth (Broderick and Merchen 1992; Obispo and Dehority 1999). However, it is difficult to decide which markers best identify microbial materials in abomasal or duodenal digesta (Broderick and

Merchen 1992; Carro and Miller 2002; Schonhusen *et al.* 1995). The methods of total purine (Zinn 1986) and incorporation of ^{15}N (Broderick and Merchen 1992; Firkins *et al.* 1987a) have been recommended for use under practical conditions. In both of these procedures for estimating microbial protein in duodenal digesta, marker concentration in a bacterial sample from the rumen or duodenal contents should be determined. This marker must be present only in the microbial fraction of interest and must represent the average marker concentration of all such micro-organisms leaving the rumen (Smith 1975).

When the total protein entering the duodenum and the concentration of marker in the microbial fraction of interest is known, quantitative estimates of the input of microbial protein to the duodenum can be made. Internal and external markers commonly used to estimate microbial protein synthesis in the rumen are listed in Table 2-1.

Table 2-1. Commonly used internal and external markers for quantifying bacterial and protozoal protein (MCP) synthesis in the rumen

Type of marker	MCP fraction estimated
Internal	
DAP[1]	Bacteria
Nucleic acids	Bacteria and protozoa
RNA[2]	Bacteria and protozoa
Individual purines and pyrimidines[3]	Bacteria and protozoa
Total purines[4,5]	Bacteria and protozoa
External	
$^{15}NH_3$ [6]	Bacteria and protozoa
$^{35}SO_4$ [7]	Bacteria and protozoa

1. Czerkawski (1974), 2 Ling and Buttery (1978), 3 Schelling and Byers (1984), 4 Zinn and Owens (1982), 5 Ushida and Jouany (1985), 6 Firkins *et al.* (1987b), 7 Beever *et al.* (1974b)

Because these techniques (^{15}N and purine derivatives) will be applied in this study to compare the microbial protein production between the different dietary treatments, they will be reviewed further in the next Section.

2.10.1 Internal markers

Purine derivatives (PD)

Dietary nucleic acids are almost totally degraded in the rumen and therefore the RNA isolated at the duodenum is predominantly of microbial origin and can be used as a microbial marker (Smith and McAllan 1970). In several studies, RNA (Ling and Buttery 1978; Schonhusen *et al.* 1990), total nucleic acids (Puchala and Kulasek 1992; Ushida *et al.* 1985; Zinn 1986), or individual bases (Schelling *et al.* 1982) have been used to identify the microbial materials present in digesta. The urinary PD technique depends on the assumption that most ruminant feed sources, especially those of plant origin are generally low in purines and that any purines present are extensively degraded in the rumen. It follows therefore, that purines that reach the small intestine will have been mainly synthesized in the rumen by micro-organisms. Purine bases of these nucleic acids are absorbed and metabolized and produce PD such as xanthine, hypoxanthine, uric acid and allantoin. All of these compounds are excreted in the urine of the sheep and the goat, but xanthine and hypoxanthine are almost absent from cattle urine because of the action of xanthine oxidase that converts these compounds to uric acid. Uric acid is excreted as such or is converted to allantoin (Chen *et al.* 1990b).

Total daily excretion of PD can be estimated from total daily urine output and its PD concentration. The amount of PD excreted is related to the amount of microbial purine or nucleic acid flowing to the duodenum from the rumen (Balcells *et al.* 1991; Chen 1989; Verbic *et al.* 1990). To predict duodenal PD flow by using urinary PD, it is necessary to evaluate the proportion of urinary PD derived from endogenous PD production in tissues. Chen *et al.* (1990a) concluded that an increase in the exogenous supply of purines via abomasal infusion reduces the contribution of endogenous metabolism source. They stated that when total daily purine excretion

exceeded 0.6 mmol/kg of $BW^{0.75}$, endogenous synthesis is completely suppressed in sheep. Equations that take the endogenous PD excretion into account and enable the purine entry into the small intestine to be predicted have been developed (Chen *et al.* 1990b). When the flow has been determined, the microbial DM (or N flow) can be calculated if the ratio of purine to DM (or purine to N) in duodenal digesta is known or can be estimated. . This technique is simple, urine volume is used based on daily collection or estimated indirectly and urinary PD concentrations can be determined by colorimetric analyses (Nolan and Kahn 2004).

2.10.2 External markers

^{15}N

The stable isotope of nitrogen, ^{15}N, has been used to label plant material or microbial material. The ^{15}N method has some advantages in comparison with radioisotopes such as its constant character, simplicity of usage and less contamination of tissues and wastes compared to other markers such as ^{32}P and ^{32}S (Broderick and Merchen 1992; Sadik *et al.* 1990). Limitations to using ^{15}N include its cost, time consuming sample preparation and the cost of mass spectrometric analysis. This isotope has been used in ruminant metabolism studies (Damry *et al.* 2001; Hristov *et al.* 2001). Estimated microbial N flow and CP degradation by ^{15}N-labelled bacteria have lower standard errors and estimates of correlation of degradation of CP have improved compared to the *in situ* method (Calsamiglia *et al.* 1996). Addition of ^{15}N-ammonia into the incubation environment *in vitro* enables microbes to assimilate it and become ^{15}N labelled. The ^{15}N enrichment ratio between microbes and ammonia indicates the proportion of microbial-N synthesised from ammonia-N. The ^{15}N isotope dilution technique has been used to quantify the dynamics of N in the rumen. Nolan and Leng (1972) found that more than 50% of microbial-N was derived directly from nitrogenous sources more complex than ammonia (*i.e.* non-protein NAN) when sheep were given lucerne diets. However, when these procedures are used *in vitro* or *in vivo*, the utilisation of plant NAN materials by micro-organisms was determined indirectly after ^{15}N-ammonia utilisation by microbes had been determined. Accordingly, this method gives lower

confidence in the estimate of non-protein NAN uptake by bacteria than would be obtained using a direct measure of ^{15}N incorporation from ^{15}N-labelled plant N.

2.11 Gas production and ruminal fermentation

When substrates are mixed with buffered rumen fluid under anaerobic conditions, they will be fermented by micro-organisms and produce VFAs (mainly acetate, propionate, and butyrate) and gases (CO_2 and methane). The *in vitro* gas production technique has been used for measuring the rate and extent of digestion of ruminant feeds. Some developments in equipment design, including automated pressure recording systems, and mathematical descriptions of the gas production profiles have been applied to make this method simpler and more precise. Because gas production kinetics and substrate degradation are closely correlated, this technique has been used extensively in ruminant nutrition studies (Dijkstra *et al.* 2005; Rodriguez *et al.* 2005; Van Gelder *et al.* 2005). However, although it is relatively easy to measure gas production during *in vitro* incubations, the processes that give rise to the hydrogen (H), methane and CO_2 are complex and not well understood (Bird *et al.* 1999).

Conversion of CO_2 and H to methane (CH_4) in the rumen results in a loss of available energy to the animal but the methanogenic organisms derive energy from this process for maintenance and growth (Wolin *et al.* 1997). Changes in the fraction of methane as a proportion of the total gas production does not influence the total amount of gas produced from fermentation because 1 mol of methane simply replaces 1 mol of CO_2.

The quantity of gas produced depends on the amount of OM fermented and is related to the rate of microbial synthesis (Krishnamoorthy *et al.* 1991) and also the molar proportions of the VFA produced (Opatpatanakit *et al.* 1994). The latter is related to the type of diet ingested by the animal. High concentrate diets tend to generate a higher ratio of propionate to acetate than fibrous diets (Russell and Wallace 1997) and because CO_2 is produced during acetate production but not during propionate production, the VFA molar proportions ratio needs to be considered when

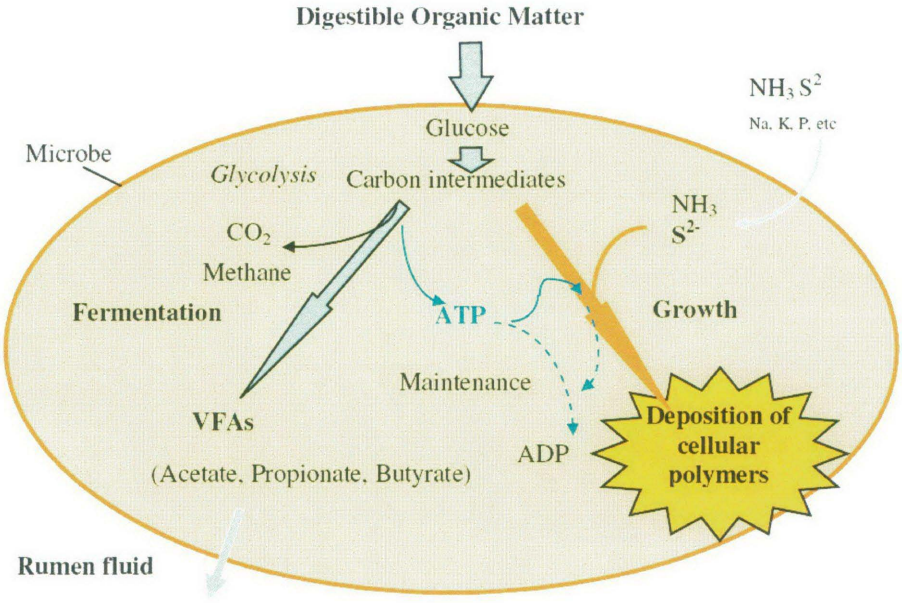


Figure 2-4. A diagram describing processes related to digestion of organic matter (left-hand arrow) and to the concurrent deposition of microbial polymers (right-hand arrow) in a rumen bacterium (adapted from Annison *et al.* 2002)

When digestible OM is fermented (via fermentation pathway) to VFA, CO_2 and CH_4, intermediates in the fermentation pathway are also removed as building monomers to be used for synthesis of cell polymers (via growth pathway). Some of the ATP produced by fermentation is used for microbial cell maintenance and any left over provides energy for synthesis of cell constituents. Other materials such as ammonia and S^{2-} are also released in the cell or are assimilated from extracellular sources to enable protein synthesis to occur. The VFA are the major sources of metabolisable energy (ME) for the host, but microbial cells also provide significant amount of ME as well as protein and other nutrients (Dijkstra *et al.* 2005).

2.13 Factors affecting digesta flow

Flow rate or rate of passage is affected by animal and diet factors and it is the amount (g/d) of digesta mass passing any point in the gastro-intestinal tract (GIT) per unit of time (Forbes and France 1993). Measuring flow rate at different points in

comparing between gas productions profiles for different substrates. These and other considerations concerning the use of this method have been reviewed by Krishnamoorthy *et al.* (2005)

2.12 Stoichiometry of OM fermentation

The stoichiometry of carbohydrate fermentation in the rumen *in vitro* has been studied for a long time (Bryant 1979; Demeyer and Van Nevel 1975). Theoretical reaction schemes, based on biochemical pathways of anaerobic carbohydrate and protein breakdown have been validated and used to predict fermentation processes (Van Nevel and Demeyer 1979).

VFAs and the gases, CO_2 and methane, are produced as by-products of carbohydrate fermentation when feedstuffs are incubated with rumen fluid *in vitro*.. Ammonia is also produced as an end-product of protein degradation but remains in solution in rumen contents and, although some ammonia is re-used for microbial protein synthesis, its rate of production can provide an indication of feed protein degradability in the rumen. VFA and gas production rates are more directly related to the rate of fermentation of OM and are therefore probably better indicators of OM degradability. The major processes of fermentation of OM and the generation and use of ATP that is used for microbial growth are shown in Figure 2-4.

the gut allows an estimation of the quantity of digestion, absorption and/or secretion in parts of the GIT.

Quantifying digesta flow throughout the digestive tract of cannulated ruminants usually involves digesta flow markers. These markers can be classified into liquid and particle associated markers. The former group includes polyethylene glycol (PEG), chromium (Cr) and cobalt (Co) ethylenediaminetetraacetic acid (EDTA) while particle associated markers include chromic oxide (Cr_2O_3), ruthenium (Ru) and ytterbium (Yb), acid-insoluble ash (AIA), lignin and n-alkanes. The characteristics of digesta flow markers are described by Faichney (1975) who indicates that markers should have the following characteristics: 1) be non-absorbable, 2) not affect or be affected by the GIT or its microbial population, 3) be physically similar to or closely related with the material it is to mark, and 4) the method of estimation in digesta samples must be specific and sensitive and must not interfere with other analyses.

Digesta flow data from numerous experiments have also been reviewed by Faichney (1993). It is clear that digesta flow is affected by level of feed intake, physical and chemical characteristics of diet and animal factors. The physiological state of the animal also affects flow rate, with pregnant and lactating animals generally having faster flow rates. He also indicated that duodenal digesta flow from the rumen of animals fed different diets can be sorted as follows: fresh forage > dried forage > chopped hay > ground hay > mixed diet > concentrate.

2.14 The use of markers to label forages

Investigations of the metabolism of forage constituents have been made by using isotope-labelled plants. In early studies, plants were labelled with $^{14}CO_2$, and the specific radioactivity curves of different C pools such as ruminal VFA, plasma, urine, and feaces were described [(Keith *et al.* 1963; Yadava *et al.* 1964) cited by Hristov *et al.* (2001)]. Application of ^{14}C is limited because it poses potential health problems, and is difficult to handle and dispose of. The radioactive isotope has been largely replaced by the non-hazardous stable isotope, ^{13}C. This isotope has potential

for studying plant metabolism in ruminants (Avice *et al.* 1996). Another option is ^{15}N, a stable isotope of N. This isotope has been used in plant physiology studies, particularly with lucerne (Glasener *et al.* 1998; Harris and Hesterman 1990). Application of ^{15}N to plants has no adverse environmental consequences.

Quantitative estimates of N metabolism in the rumen have been derived from studies in which ^{15}N was used as the tracer. The ^{15}N is usually administered intraruminally as ^{15}N-labelled urea, $(^{15}NH4)_2SO_4$, or $^{15}NH_4Cl$ to label the rumen ammonia pool and other pools connected to ammonia. Some results of such studies, especially those that have quantified rumen ammonia metabolism and the ammonia contribution to microbial protein synthesis, have been reported in Section 2.8.2.

Current understanding of plant N transactions among various N pools in the rumen has been enhanced by the use of ^{15}N-labelled feed. However, there have not been many *in vivo* studies using this technique, probably because of the costs involved and the time needed to produce the ^{15}N-labelled plant in the amounts needed. In the rumen, the ^{15}N from the labelled plant will appear in various rumen N pools at rates depending on the original distribution of the ^{15}N in plant N and on the rates of plant degradation. Because ammonia is the end product of degradation of N-containing substances, the appearance of ^{15}N label in this pool can then be used to estimate the degradation of plant N materials (Chapman and Norton 1984). However, even if the plant N materials are completely degradable in the rumen, all of the ^{15}N present in the plant will not necessarily enter the ammonia pool; some ^{15}N may enter peptide and amino acid pools and be taken up by bacteria without passing through ammonia. For such studies, it is preferable to use ^{15}N-ammonia and ^{15}N-labelled feed in one experiment in animals subject to closely similar conditions (Hristov *et al.* 2001). In this case, net flux (irreversible loss) of ammonia obtained from the ^{15}N-ammonia can be assumed to be applied when ^{15}N-labelled feed is used as the source of the tracer. The ratio of areas under primary and secondary enrichment v. time curves indicates the fraction of the N in the secondary pool that originated from the primary pool. So, for example, the fraction of N in rumen microbes that was derived from rumen ammonia, sometimes referred to as the transfer quotient (TQ)

can be determined. In this example, (1-TQ) is the fraction of N present in bacteria assimilated from NAN sources such as peptides and amino acids.

2.15 Implications from Literature Review

The extent of degradation of dietary protein in the rumen has been extensively investigated by numerous workers, mainly using *in vitro* or *in situ* techniques. These studies have provided functional information about the degradation of carbohydrates and proteins of feedstuffs in the rumen. The feed protein degradation products that are assimilated by rumen micro-organisms have also been investigated, but mainly in batch cultures of rumen micro-organisms or in continuous fermenters. Models describing the kinetics of feed N in the rumen and the whole animal have been developed, but the information on which they have been based has generally been derived from animals given pasture plants preserved as hay or silage, or from supplementary feeds such as cottonseed meal and soybean meal or from chemicals such as urea. Only a few studies of feed protein degradation have been made *in vivo*: an even smaller number have provided information for model building from grazing animals or confined animals given 'cut and carry' forages.

For these reasons, the extent of assimilation of protein degradation intermediates (peptides and amino acids) *in vivo* by microbial populations in the rumen of animals given fresh forages is not yet clear. There is still uncertainty about the conditions that facilitate peptides/amino acids uptake and about whether higher concentrations of these materials in rumen fluid leads to higher efficiencies of microbial growth. Accordingly, the experimental work in this thesis has focused on the digestion characteristics of fresh forages processed in different ways.

Chapter 3 General Materials and Methods

3.1 Introduction

This Chapter outlines the materials and methods that were used in more than one of the experiments reported in this thesis. Specific details about materials and methods relevant to an experiment are provided in the relevant chapter. The experimental work undertaken was approved by the Animal Care and Ethics Committee (AEC) of the University of New England according to the legislation of the Animal Research Act (1985) and Regulations (1990).

3.2 Experimental procedures

3.2.1 Chemical analysis of forages

3.2.1.1 Dry matter (DM)

Dry matter was determined by weighing approximately 15-20 g of sample into a pre-weighed dry aluminum tray and the combined weight was recorded. The aluminum tray containing the sample was then placed in a fan forced oven for 24 h at 105°C. After removal, the sample and tray were transferred to a desiccator and allowed to cool. The combined sample and tray were weighed and recorded and the DM% was calculated according to the following equation:

sample represent the real rumen contents. Rumen samples in the space between the plastic layer and steel pipe was transferred to a 50 ml plastic bottle and stored frozen at -20 °C for subsequent analysis of N concentration and ^{15}N enrichment. The DM and N content of whole rumen samples were 12.6 and %3.8, repectively and were analysed (see Sections 3.2.1.1and 3.2.8.2) to provide evidence that these samples can be considered as whole ruminal contents.

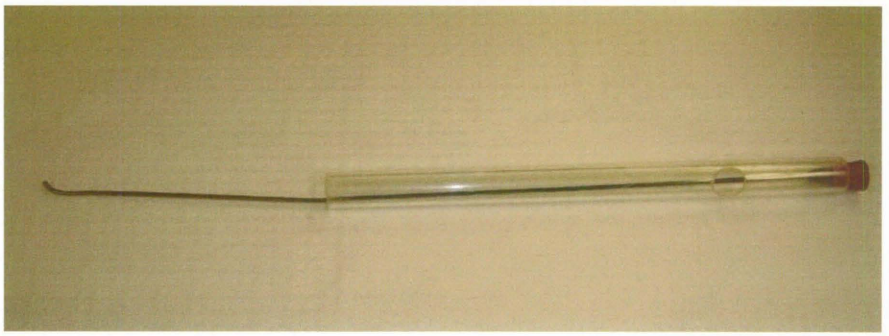


Figure 3-1. Device used to collect whole rumen contents

3.2.3 Measurement of digesta flow

In the experiments described in this thesis, acid insoluble ash (AIA) and Chromium ethylenediaminetetraacetic acid (Cr-EDTA)(Downes and McDonald 1964) were used as non-absorbable solid and liquid phase markers, respectively. A double-marker method proposed by Faichney (1975) was used for correcting sampling errors and composition and flow of the digesta passing the sampling point

Continuosly infusion of Cr-EDTA solution into the rumen through polyethylene silastic infusion lines with an internal diameter of 1.5 mm was started 48 h before sampling. The infusion was made using a peristaltic pump and the amount of infused Cr-EDTA for each sheep was calculated by subtracting the mass of the reservoir at the end of each infusion day from the mass at the start of the day. It is supposed that under steady state conditions, the rates of infusion of Cr and AIA entering to the rumen should be equal to the amount of that leaving the rumen and passed with the true digesta through to the abomasum (Faichney 1975). Any difference in the ratios of AIA and Cr in the digesta to the ratios in the infusates

$$DM\,(g/kg) = \left(\frac{(combined\ \ dry\ \ wt) - (tray\ \ wt)}{(combined\ \ wet\ \ wt) - (tray\ \ wt)} \right) \times 100 \qquad (2)$$

3.2.1.2 Organic matter (OM)

The sample assessed for OM was transferred to a crucible tray and placed in a muffle furnace for 30 min at 350°C and then for 2 h in 600°C. The combined weight of the resultant ash and crucible was recorded and used to estimate the sample OM content as follows:

$$OM(g/kg\,DM) = 100 - \left(\frac{combined\ \ ash\ \ wt - crucible\ \ wt}{combined\ \ dry\ \ wt - crucible\ \ wt} \times 100 \right) \qquad (3)$$

3.2.1.3 Starch, water-soluble and non structural carbohydrates

The starch, water soluble carbohydrate (WSC), and non-structural carbohydrates (NSC) content of fresh forages were analysed as a service by University of Sydney, Camden. Soluble carbohydrates (SC) were extracted from forage with water. The resulting filtrate was heated with anthrone in sulphuric acid to form a blue-green complex. The absorbance of the extract was measured on a spectrophotometer and the value obtained used to read the concentration of water-soluble carbohydrates from a standard calibration curve (see Appendix 1 for a more description of the anthrone method).

3.2.2 *In vitro* experiments

3.2.2.1 Whole rumen content sampling

Whole rumen samples were collected from the sheep used in the experiments described in Chapter 6 with a core sampling probe (20 mm internal diameter, 350 mm long with a rubber bung at the bottom; Figure 3-1). The probe consisted of a thin steel pipe with plastic end which was covered with a layer of round plastic. The sampler was moved to different sides and caution was taken to make sure the taken

indicates that the sample of digesta was not representative of true digesta. Prior to collection, any abomasal digesta trapped in the abomasal cannula stem were scraped out and discarded. A John vial was placed underneath the opened cannula and digesta were allowed to flow into it. The collection was at times aided by slowly inserting a smooth plastic tube into the cannula and gently swirling the abomasal digesta. The digesta were stored in the collection container at –18 °C until analysis.

Fractions of both markers collected via the abomasal cannulae in digesta separated into liquid and solid fractions is described in Section 3.2.3.1. True digesta were reconstituted mathematically by appropriately re-mixing the two components to contain equal fractions of both markers. Cr and Co-EDTA infusion solutions and abomasal digesta were subjected to Cr and Co analysis following a perchloric acid ($HClO_4$) / hydrogen peroxide (H_2O_2) digestion. The Cr and Co concentration were analyzed on an Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Varian, Model MPX).

3.2.3.1 Sample preparation and digestion

The abomasal digesta samples were divided into liquid and solid fractions by leaving samples after thawing to stand at room temperature for 15 min. The upper layer was transferred into another container (liquid phase sample) and the solids-rich phase (deposited part which includes mostly solid) was left in the original container. About 0.2 g (± 0.01 g) or 2 ml of liquid sample were weighed into previously weighed Schott bottles and 2 ml of 7:3 (v/v) mixture of $HClO_4$ (70%) and H_2O_2 (30%) (prepared fresh daily) was carefully added in a scrubbed fume cupboard. The cap was replaced but left on loosely. Samples were predigested for at least 2-3 h at room temperature. One ml of H_2O_2 was added, the cap was tightened and the bottle was placed in an oven at 80°C for 30 min. After the bottles cooled, a further 1 ml of H_2O_2 was added and the bottles were capped tightly and digested for 1 h in an oven at 80°C. After cooling, samples were diluted (1:24) with deionised water. The solution was mixed thoroughly and allowed to stand at least for 2 h; it was then filtered through No.1 Whatman paper into a glass vial with a positive snap-on cap and brought to the ICP-OES room 24 h before the analysis. The analysis was done on

3 spectral lines and the best line was selected on the basis of highest recovery of known standards.

Standard solutions containing 1 g of Co in 0.5 mmol HNO_3 and 1 g of Cr were available. A series of standards containing 1, 2, 4, 6, 8 µg/ml of Cr and Co were prepared by diluting them in 5.6% (v/v) $HClO_4$ solution. The quantitative recovery of Cr and Co during digestion of some of the samples was checked by spiking them with known quantities of Cr and Co standard solutions. Recovery data are presented in Appendix 4.

3.2.3.2 Determination of AIA

The AIA concentration in ground (1 mm sieve) feed samples and the solids-rich part of abomasal digesta was determined as described by Choct and Annison (1990). Approximately 2 g of feed or 1 g of digesta was weighed into a tared filtered crucible (porosity 4) and dried in a forced-draught oven at 105°C for at least 8 h. The mass of DM remaining was recorded and the sample was then ashed at 480°C for at least 8 h. The sample was allowed to cool and transferred to an evaporating dish. Slowly, 4M HCl was dispensed into the evaporating dish until the sample was wetted from underneath. The crucible was then filled to about 75% of its volume with the 4 M HCl. The evaporating dish was placed on a hot plate and HCl was allowed to boil for about 15 min. After cooling, the crucible was taken out and HCl was removed by suction, rinsed with the same 4 M HCl and then with deionised water. The sample was dried in an oven at 105° for 2 h after which the ashing and removal of the acid soluble ash was repeated. The sample was finally dried in an oven at 105°C for 6 h and weighed after cooling in a desiccator.

3.2.4 Preparation of rumen fluid and isolation of bacteria

A 15 ml sample containing bacteria (*e.g.* rumen fluid or rumen digesta) was first centrifuged (20,000 *g*, 15 min at 4°C; Beckman Model J2-21M) and the supernatant was carefully transferred into a wide-neck McCartney bottle, preserved with 18M H_2SO_4 and stored at −18°C until analysed. Bacteria were isolated from the

residue by twice re-suspending the residue in 20 ml deionised water, re-centrifuging and removing the top (bacteria-rich) layers of the residue. The final bacterial isolate was suspended in about 3 ml deionised water and stored at -18°C for analysis of bacterial-N and ^{15}N enrichment (see Section 3.2.8.7). Bacterial pellets were observed under a light microscope to be free of feed particles and protozoa.

3.2.5 Urine and faeces analysis

3.2.6 Urine

The metabolism crates used in the studies described in Chapter 6 were equipped with a mesh separator which enabled urine and faeces to be collected separately. A complete collection of urine from the sheep was made on a daily basis over a period of 4 d. To prevent bacterial degradation of allantoin and volatile N losses, urine was collected into the bucket containing 50 ml of 10% (v/v) H_2SO_4. Because of the variability in the urine volume from different sheep, the urine was tested daily to ensure the pH was maintained below 3.0 and the volume of H_2SO_4 added to the collection containers was varied accordingly. The 24 h urine collections were diluted by adding tap water to a final volume of 2 L. After thorough mixing, a 50 ml sub-sample of the diluted urine was stored in a plastic bottle at -18 °C.

3.2.6.1 Analysis of allantoin concentration in diluted urine

Allantoin was analyzed according to the method described by Chen and Gomes (1992). The quantity of microbial purine entering the small intestine (X, mmol/d) was estimated from the excretion rate of PD in urine (Y, mmol/d) using the equations for sheep proposed by Chen *et al.* (1990a) and Chen and Gomez (1992);

$$Y = 0.84X + (0.150\ kg^{0.75}e^{-0.25X}) \qquad (4)$$

Flow of microbial N (MN) (g/d) into the intestine based on total PD excreted in urine was then estimated by using the following equation:

$$MN/d = 70X/ (0.116) (0.83) (1000) = 0.727X \qquad (5)$$

where X is the intestinally absorbed PD and Y is the urinary excretion of PD in mmol/d. The validity of this relationship is based on the accepting the following assumptions that digestibility of infused purine is about 0.83. The N content of purine is about 70 mg/mmol and the ratio of purine N: total N of mixed rumen bacteria is 11.6 to 100. In this study, estimation of the microbial N supply was based on the mean rate of excretion of allantoin during a 4 d period. In sheep ideally all four PD (hypoxanthine, xanthine, uric acid and allantoin) should be determined, but allantoin normally forms the highest proportion (Kahn 1996).

3.2.7 Faeces

Faeces were collected in plastic bags for at least 4 d over the experimental period. After collection, the faeces were transferred into pre-weighed drying trays and the combined weight was recorded. They were then placed into an 105 °C forced-draught oven for 48 h. On removal, the dry combined-weight was recorded and used to calculate the total faecal DM output (g DM/sheep/d). Faeces were sub-sampled and milled through a 1 mm sieve for N analysis.

3.2.8 Laboratory analysis

3.2.8.1 Analysis of VFAs

Total VFA concentration and the molar proportions of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acids were determined on the rumen fluid supernatant according to the method of Erwin *et al.* (1961) using a gas liquid chromatograph (Model 427, Packard Instrument Co., USA). Iso-caproic acid was used as an internal standard. The gas chromatograph was connected to a recording data processor (Model 604, Packard Instrument Co., USA).

3.2.8.2 Total N

Total-N content of feed samples was determined on finely ground sample DM (1 mm sieve) either using an N analyzer (Leco FP 2000) or, when N was

required for ^{15}N analyses, by micro-Kjeldhal digestion followed by steam-distillation and titration of the resulting ammonia.

3.2.8.3 Ammonia-N

The concentration of ammonia-N was determined by steam-distillation of samples contained ammonia using saturated Na-tetraborate to make the solution alkaline (around pH 9), followed by titration. In this method, a sub-sample of nitrogenous material, such as rumen fluid supernatant (3 ml) or soluble protein supernatant (2 ml) plus few drops of silicone anti-foaming solution and 4 drops of universal indicator, to prevent foaming and to give a colour indication of the pH was added to a micro-Kjeldhal digestion flask. The flask was attached to a distillation apparatus. By adding 6-7 ml of saturated Na-tetraborate, the pH of sample was made to about pH 9 and then immediately steam-distilled for 3.5 to 4 min. The volatile ammonia was collected into a beaker containing 3 ml 0.025 M (standardized) H_2SO_4. The distillate was continuously stirred and titrated to pH 5.0 with standardized 0.025 M NaOH (Autoburett ABU80, Radiometer, Copenhagen). Recovery of ammonia N was checked by distilling 1 ml of standard $(NH_4)_2SO_4$ and blank samples (3-4 ml deionised water). Distilled ammonia was captured in the beakers contained 3 ml of 0.025 M H_2SO_4. After distillation of each pair of samples, in order to reduce the risk of ^{15}N contamination, 15-20 ml of ethanol was distilled in both distillation flasks for about 5 min to remove ammonia adsorbed on the glass surfaces.

3.2.8.4 Preparation of samples for ^{15}N analysis

After steam-distillation and titration, the samples containing ammonium sulphate $(NH_4)_2SO_4$ were further acidified with 1 ml of 0.025 M H_2SO_4 and dried in an oven at 85°C. A minimum amount of deionised water (about 1-2 ml) was added to the distillation beaker to dissolve the dried salt and then the solution was transferred to a 5 ml vial and dried in an oven at 85°C. The dried $(NH_4)_2SO_4$ was re-dissolved in 100 µl of H_2O. An appropriate volume of the resulting solution (to contain 80-100 µg N) was carefully pipetted into an 8 x 5 mm tin capsule (Elemental Microanalysis Ltd., Cat. No. D1008) and dried in a desiccator overnight. A very small piece

(around 2 mm × 2 mm) of filter paper (Whatman, no. 541) was added to each tin capsule to absorb the solution and to prevent creeping of the sample over the edges of the capsules during drying. Evacuation of the desiccator was done carefully to prevent splashing of sample solution in the capsules. The next day, capsules containing the dried salt were closed using tweezers and then folded to make them small enough to fit into the sample carousel of an automatic N/carbon/sulphur analyser (Carlo Erba Instruments; Model NA1500).

3.2.8.5 Oxidation of samples and estimation of ^{15}N abundance

The samples were loaded in a series that started with 3 blank capsules, followed by 2 conditioning samples (natural abundance) followed by four reference standards (wheat flour, natural abundance). The same reference standards were placed after the last sample of each analytical run. Any drift in the estimates of sample abundance resulting from changes in the analytical conditions within the mass spectrometer during that analytical run could then be detected, and allowed for, by assuming the drift was linear over time. The squashed tin capsules contained samples were dropped automatically by the N analyser into a furnace, filled with pure O_2 at 1030°C, where the ammonium salt was oxidised by flash combustion to N_2 gas. The resulting N_2 gas was then dried and passed into the analyser tube of an isotope-ratio mass spectrometer (Tracermass; Europe Scientific) and its ^{15}N abundance was determined.

3.2.8.6 ^{15}N enrichment analysis and verification

Measurement of ^{15}N enrichment by isotope-ratio mass spectrometer was an important laboratory analysis carried out in this study. The ^{15}N enrichment on different nitrogenous rumen samples was determined before and after administration of ^{15}N sources to give a better understanding of the kinetics of N between different pools in the rumen. Therefore, it was necessary that sample preparation procedures for ^{15}N analysis of purified samples were verified throughout the study. The samples used for ^{15}N analysis must be pure samples of the N specific to the material being investigated (*e.g.* rumen bacterial N must not be contaminated with ^{15}N-ammonia)

and it must be delivered to the mass spectrometer without contamination by extraneous N during preparation. Cross-contamination with ^{15}N between samples which might occur by absorption of ^{15}N on glassware or distillation apparatus must be prevented. The necessary precautions that were followed in this thesis are discussed in detail by Nolan (1971). Accuracy and repeatability of ^{15}N analysis over time were examined (see Appendix 3).

3.2.8.7 Bacterial samples enrichment

The final bacterial isolates were stored at -18°C and then freeze dried. Then, 2-3 glass balls were added to each tube containing the bacterial pellet and the tubes were vortexed (MS2, mini-shaker, In vitro Technologies, Pty Ltd) for 2 min at 2200 rpm. Finally about 1 mg of bacterial powder was added to the tin capsules (Elemental Microanalysis Ltd., Cat. No. D1008) and delivered to the mass spectrometer for determination of ^{15}N enrichment in bacterial samples (see Section 3.2.8.5).

3.2.8.8 Soluble protein

After centrifuging fluid samples (20,000 g, 15 min, 4°C; Beckman Model J2-21M), a sub-sample of each supernatant (about 8 ml) was added to a pre-tared disposable tube containing 0.8 ml 65% (w/v) trichloroacetic acid (TCA) solution to precipitate soluble proteins. The exact mass added was determined by weighing the tube before and after the addition of fluid. The tube was well mixed and stored at 4°C for at least one day, to facilitate complete protein precipitation, after which the tube contents were transferred to another centrifuge tube and centrifuged at (18600 x g, 15 min, 4°C). The supernatant was kept at -18°C for later analysis to determine peptide and amino acids but the pellet was suspended in 10 ml 0.025 M HCl and again centrifuged at (20,000 x g, 15 min, 4°C). The HCl washing solution was carefully decanted to waste, and the washed protein precipitate was freeze-dried. Upon removal from the freeze-drier, the tubes were capped and the dried material was weighed into tin capsules for later analysis for protein N and ^{15}N concentration by Mass-Spectrometer.

3.2.8.9 Peptide and amino acid concentrations and enrichments

Samples of the protein-free supernatant (4 ml) from the soluble protein analysis (Section 3.2.8.8) were distilled about 3 min with Na tetraborate solution (to have the solution pH about 9) to remove ammonia, leaving a non-protein, NAN fraction (presumed mainly peptides and amino acids). The latter fraction was then subjected to Kjeldahl digestion to convert all N to ammonia which was then isolated by steam distillation as described in Section 3.2.8.3. Protein-free supernatant (4 ml) was subjected to Kjeldhal digestion for approximately 3 h at 320 °C or until the solution became clear. A tablet of low selenium catalyst was added to each flask followed by 3.5 ml of 18 M H_2SO_4. After cooling, approximately 10 ml of H_2O was carefully added to increase the volume and the sample was sealed, left overnight and ammonia was distilled from the contents the following day. Before the contents were distilled, anti-foaming solution and universal indicator were added. The contents were then made alkaline by slowly adding approximately 10 ml of 40% (w/v) NaOH (to over-neutralize the H_2SO_4 from the Kjeldahl digestion) and steam-distilled to release ammonia-N which was retained for ^{15}N analysis as described in Section 3.2.8.4.

Chapter 4 Degradation of fresh and frozen-thawed ^{15}N-labelled lucerne and ryegrass in rumen fluid *in vitro*

4.1 Introduction

There is little information on rumen fermentation characteristics of fresh forages for use in ruminant feeding systems (Gosselink *et al.* 2004b). Lucerne and ryegrass are common pasture species grazed by Australian livestock and more detailed information about their rumen degradabilities would be beneficial. Current ruminant feeding systems depend on knowledge of intake and composition of feeds being ingested and the rate and extent of degradation of feed OM and protein in the rumen. Fermentation characteristics of feeds in rumen fluid can be studied by *in vivo*, *in situ* and *in vitro* techniques.

The protein value of feeds for ruminants is based on quantifying both the amount of dietary and microbial protein supplied to the small intestine. It is difficult, however, to study fresh forages in ruminant feeding experiments because it is necessary to have both the animal and the fresh forage samples ready at the same time. To determine nutritional quality of forages, samples are often collected in the field prior to testing and different preparation methods such as freezing, freeze-drying or oven-drying are often used (Cone *et al.* 1996; Noziere and Michalet Doreau 2000; Parissi *et al.* 2005). However, digestive characteristics of dried or frozen samples may differ from those of forages when they are grazed by animals. In addition, the method used to prepare samples for incubation can affect the results,

e.g. cutting fresh forages to smaller pieces or mincing before incubation to more closely simulate the size and characteristics of the forages after chewing by animals.

The aim of the experiment reported in this chapter was to determine the ruminal DM and protein degradabilities of lucerne and ryegrass using an *in vitro* method. Fresh (F) or frozen-thawed (FT) ^{15}N-labelled plant material was incubated. Volatile fatty acid (VFA), gas production, and N recovery in ammonia and microbial crude protein (MCP) were measured to determine if degradability differed between species of forage or between methods used to prepare the forages before incubation.

4.2 Materials and Methods

4.2.1 ^{15}N labelling and freezing of forages

Lucerne (*Medicago sativa* L.) and perennial ryegrass (*Lolium perenne* L.) were grown in 4 L pots in a glasshouse. The potting mix was almost free of N (see Table 4-1) and the required N for plant growth was provided from either fertilizer (8 g of Multigro fertilizer (N= 10.1%, P= 3.5%, K= 5.5%, S= 16.3%, Ca=7.8%)) (non-labelled) added monthly, or from the ^{15}N provided by hand watering with $^{15}NH4^{15}NO_3$ solution twice weekly (14 mg of ^{15}N per pot per month, 99.2% enriched, Shanghai Research Institute of Chemical Industry). The level of ^{15}N enrichment of the plants was tested every 4 weeks and, when required, N fertilizer was applied. Figure 4-1 shows fresh lucerne and ryegrass growing in a glass house.

Table 4-1. Potting mix composition

Component	Composition
Macroingredients (% vol/vol)	
Sand	50
Soil	25
Peat Moss	25

Figure 4-1. Fresh lucerne and ryegrass in glass house

Second cut lucerne (pre-blooming, 17% DM, 26% CP) and ryegrass (4th leaf stage, 13% DM, 18% CP) were incubated *in vitro*. Samples of each forage species were cut 5 cm above the soil level and thoroughly mixed then sub-sampled. One sub-sample was placed in sealed plastic bags and stored at -20 ºC for 24 h. Next morning, on the experimental day, the fresh sample was cut similarly about 30 min before incubation and chopped like frozen sample. The plastic bags containing the frozen forages were opened, and the contents were spread out and left on laboratory bench at room temperature for about 30 min before start of incubation.

4.2.2 Rumen fluid collection

Rumen fluid (approximately 600 ml from each sheep) was obtained 1 h before feeding from 4 Merino sheep fitted with rumen cannulas and used as inoculum. The collected rumen fluid was then brought to the laboratory in an anaerobic 5 L Schott bottles (filled with CO_2 for about 2 min) placed in a thermos container filled with water at about 40 °C. The sheep were housed indoors in individual metabolism cages in an animal house at the University of New England.

Each sheep had continuous access to fresh drinking water and received a basal diet of lucerne (600 g/d, 92% DM, 19% CP) and oaten chaff (400 g/d, 91.5% DM, 7.5% CP). New feed was offered once a day at about 1000 h.

4.2.3 Measurement of gas production

Gas production was measured *in vitro* by using 1 L incubation flasks held in a shaking water bath, set at 39°C during incubation. Bottles were connected by plastic tube to a 500 ml measuring cylinders positioned upside down in a square bucket containing water (see Figure 4-2**Error! Reference source not found.**). At the beginning of incubation, the cylinders were completely filled with water and the quantity of gas produced during the incubation was determined by a liquid displacement technique. The cylinders were refilled with water when necessary and measurements continued.

Figure 4-2. Apparatus used for *in vitro* incubation of fresh and frozen-thawed forages with rumen fluid

4.2.4 *In vitro* procedure

The *in vitro* incubation was conducted in duplicated 1 L incubation bottles at the ruminant digestion laboratory (W32, University of New England). The rubber stopper of each bottle was equipped with a Bunsen valve that was connected through a silastic tube to an inverted glass measuring cylinder for reading gas production during the incubation. The forage species were prepared for incubation as fresh (F), frozen-thawed (FT) or frozen-thawed + 1 g maize starch (85652 Fluka, Biochemica) (FTS).

Approximately 5 g DM of F and FT lucerne and ryegrass were added to each incubation bottles and, for the FTS treatments, 4 g DM of FT lucerne and ryegrass plus 1 g starch were added to incubation bottles. Each species x preparation treatment sample was mixed with 300 ml McDougall's buffer solution (pH 7 ± 0.1; see Table 4-1) and macerated for 30 sec using a blender set at high speed. The slurry transferred directly to the incubation bottles. Two hundred ml of the rumen fluid inoculum was strained through cheese cloth and gassed with CO_2 and then 200 ml added to each of the incubation bottles containing the samples. The tubes were filled with CO_2 and sealed. Blank bottles containing only rumen fluid and buffer were also prepared in the same manner.

Table 4-2.Chemical composition (g/L) of McDougall's buffer solution

Chemical	Concentration (g/l)
$NaHCO_3$	9.76
Na_2HPO_4	2.8
NaCl	0.47
KCl	0.57
$CaCl_2$	0.04
$MgCl_2.6H_2O$	0.13

The blanks and the samples in the glass bottles were incubated in shaking warm water bath (39°C) for 12 h. Samples of inoculum were taken without opening the bottles at 0, 3, 6, 9 and 12 h using a sampling syringe. The samples taken at zero time were placed into a container of crushed ice to reduce the rate of fermentation but it seems that microbes remained active for at least 30 min. Accordingly, these samples were assumed to represent fermentation in the first 30 min of incubation. Fluid was taken from each incubation vessel into the sampling syringe and returned to the incubation bottles, this action was repeated at least three times and finally 20 ml was removed and transferred to a 30 ml centrifuge tube and placed in crushed ice. The pH of each sample was recorded immediately (Eco scan pH 5/6, Eutech instruments) and the remaining fluid was then centrifuged (20,000 *g*, 15 min, 4°C). The supernatant fraction was acidified with 0.25 ml 18 M H_2SO_4 and stored at -18°C for analysis of ammonia-N. Bacteria were separated from the solid fraction but the amounts separated were too small to enable the N concentrations and enrichments to be determined.

4.2.5 Chemical methods

After thawing, stored incubation fluid supernatant fractions were analyzed for ammonia (Section 3.2.8.3) and total VFA concentrations and proportions (3.2.8.1).

4.2.6 Calculations

Ammonia accumulation during the incubation period was calculated as the ammonia-N present in the flask with added sample (concentration x volume) minus the concentration of ammonia present in the blank flask at each time of sampling. Total production of VFA and gas from samples (mmol/g OM) was similarly adjusted for the blanks. Additional information about the fermentation and microbial growth was obtained by predictions based on stoichiometry. It was assumed that growth of cells was not limited by an insufficiency of carbon intermediates or ammonia or minerals or other essential nutrients. Specifically, ATP generation was calculated from the quantities of VFA produced by assuming that 2, 3 and 2 mol ATP would be generated during the synthesis 1 mol acetate, 1 mol propionate or 1 mol butyrate, respectively (Czerkawski 1986). The quantity of OM fermented (represented by moles of anhydro-glucose) to produce the VFA in the incubation was calculated from balanced chemical equations (Czerkawski 1986, see appendix 2) by multiplying the moles of acetate, propionate, butyrate and valerate produced by 0.5, 0.5, 1, 1, respectively and summing the results. Microbial DM was calculated by multiplying the amount of ATP produced by Y_{ATP} (10 g DM/mol ATP; Bauchop and Elsden, 1960) and the quantity of MCP produced was calculated by assuming MCP was 50% of microbial cell DM. Gas production was calculated with the following formula (Cone and van Gelder 1999):

$$Gas(ml) = V_m\left(H_A + 2H_B + 0.87H_T\right) \qquad (6)$$

where V_m is the molar gas volume, which is 24 l mol/l at 20°C, H_A is acetic acid, H_B butyric acid and H_T is the total amount of VFA (mmol/g OM).

Cumulative total VFA production, percentage of total N recovery (as ammonia and MCP) and fraction of OM fermented are fitted using the equation proposed by (Orskov and McDonald 1979).

$$y = a\left(1 - e^{-bt}\right) \qquad (7)$$

Where *a* is the fraction potentially degradable and *b* is the fractional rate of fraction *a*.

4.2.7 Statistical analysis

Data were analyzed as a 2×3 factorial design using repeated measures with the MIXED procedure of SAS v. 9.1.3 (SAS 2003). There were two forage species (S): lucerne (L) and ryegrass (R) and three preparation methods (PM) i.e. fresh, frozen-thawed and frozen-thawed+ starch. The statistical model used was:

$$Y_{ijk} = \mu + S_i + PM_j + (S.PM)_{ij} + T_k + \varepsilon_{ijkl}$$

where i = species (L, R); j = method of sample preparation (F, FT and FTS); Y_{ijk} = observation k in level of i of factor S and level j of factor PM; S_i = the effect of level of i of factor S (species); PM_j = the effect of level of j of factor PM; $(S.PM)ij$ = the interaction between level of i of factor S with level j of factor PM; Tk = time of incubation; $\varepsilon ijkl$ = random error with mean 0 and variance σ^2.

4.3 Results

4.3.1 Cumulative total VFA production and proportions

The effects of S and PM on cumulative TVFA are illustrated in Figure 4-3.

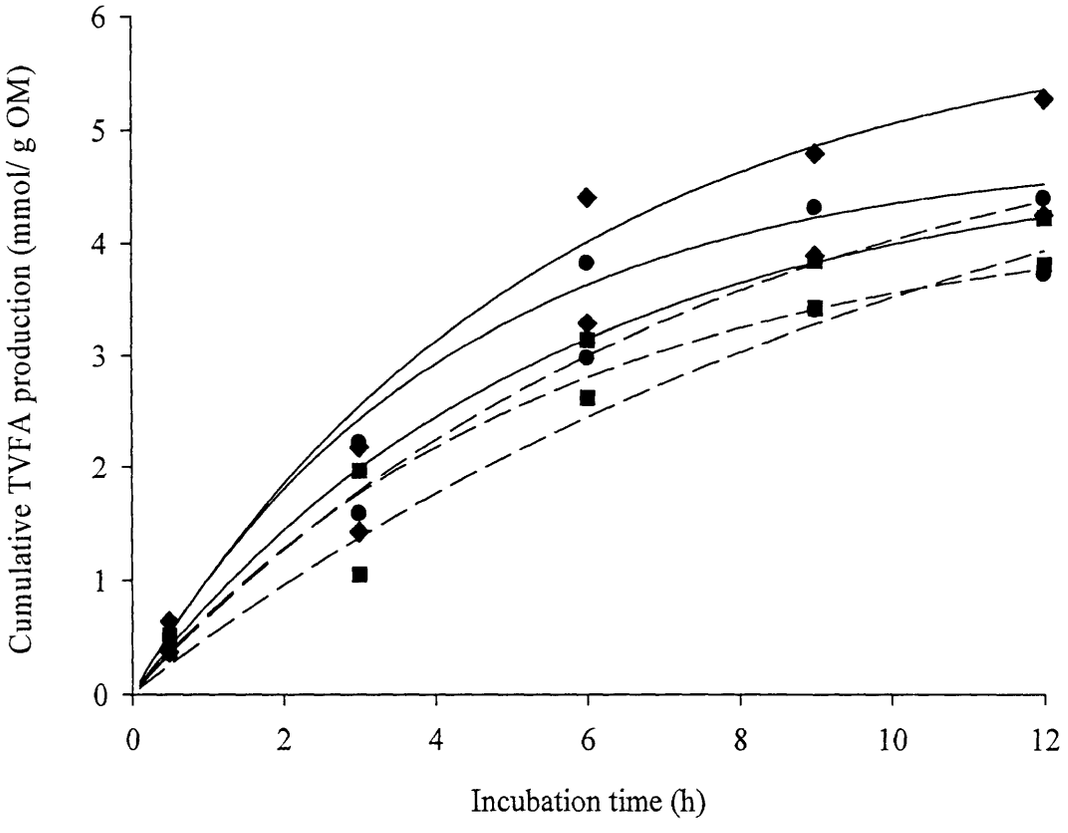


Figure 4-3. Fitted cumulative *in vitro* total VFA production in vitro (mmol/g OM) in lucerne (—) and ryegrass (---) for each method of sample preparation: fresh (♦), frozen-thawed (■) and frozen-thawed + starch (●) at different incubation sampling times.

The TVFA production at the end of incubation was higher ($P<0.01$) for lucerne than for ryegrass (4.63 L *v.* 3.93 R mmol/g OM ± SEM 0.03). There was also a significant ($P<0.01$) effect on cumulative TVFA due to PM (4.76 F *v.* 4.02 FT *v.* 4.06 FTS mmol/g OM ± SEM 0.04). Incubation time had a significant ($P<0.01$) effect on cumulative TVFA. The interaction between S and PM was significant ($P<0.01$) as were the interactions between S and T, and PM and T ($P<0.01$). However, the 3-way interaction between S, PM and T was not significant ($P=0.31$). The effect of S and PM on acetate: propionate ratio (C2:C3) at different incubation times is shown in Table 4-3.

Table 4-3. Mean values of acetate to propionate ratio (C2:C3) at different times after incubation *in vitro*

	Lucerne			Ryegrass				*P values*		
Preparation method	F	FT	FTS	F	FT	FTS	SEM	S	P	S*P
Incubation time (h)										
3	2.30[a]	2.46[b]	2.06[a]	2.16[a]	3.37[c]	2.21[a]	0.12	NS	<0.01	<0.01
6	2.58[a]	2.59[a]	2.34[a]	2.41[a]	2.76[b]	2.30[a]	0.12	NS	<0.01	<0.01
9	2.65[a]	2.61[a]	2.50[a]	2.54[a]	2.86[b]	2.31[a]	0.12	NS	<0.01	<0.01
12	2.86[a]	3.03[b]	2.66[a]	2.89[a]	3.14[b]	2.63[a]	0.12	NS	<0.01	<0.01

Means within a row without a common superscript differ (P<0.05)

As shown in Table 4-3, there was no significant difference between species in C2:C3. However, this ratio was significantly higher ($P<0.01$) in frozen-thawed than in other treatments. The interaction between forage species and method of sample preparation was also significant ($P<0.01$). In Figure 4-4, pH values in each incubation bottle over time are presented.

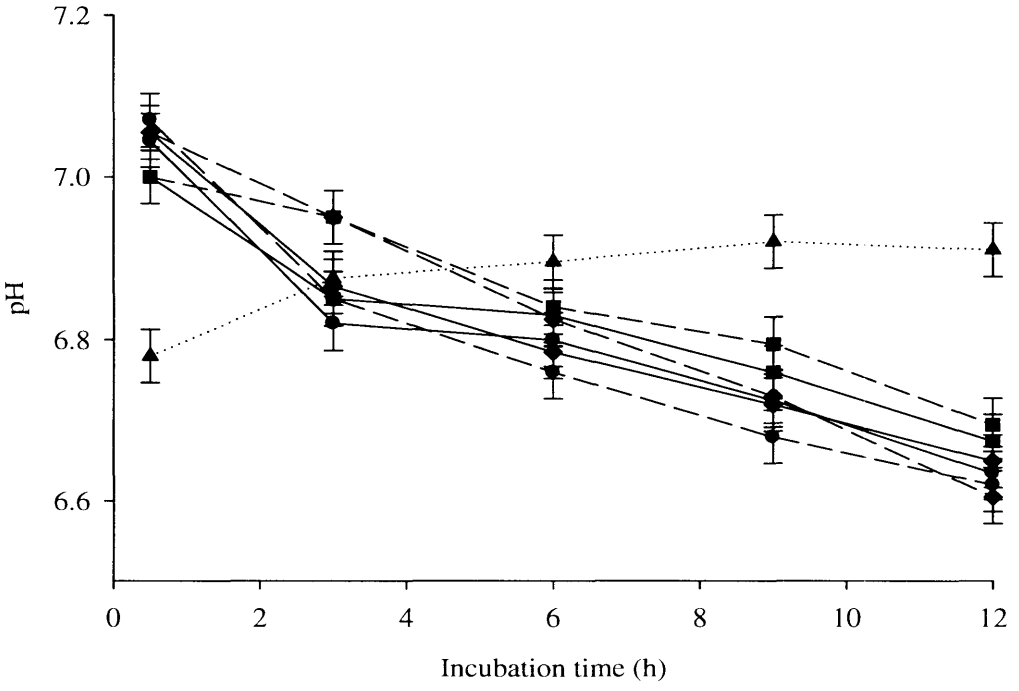


Figure 4-4. pH changes in incubation bottles containing lucerne (—) or ryegrass (---) prepared as fresh (♦), frozen-thawed (■) or frozen-thawed + starch (●), and blanks (rumen fluid and buffer only) (···) at different incubation sampling times (Means ± SEM).

As incubation progressed, pH decreased significantly in the flasks containing forage samples but increased in the blank incubations (rumen fluid and buffer only). The differences between pH in the incubation bottles were significantly different between bottles containg fresh forages. However, the changes in pH were less than 0.4 pH units in all flasks during the 12-hour incubations.

4.3.2 Organic matter fermentation

The percentage of the OM added to the flask that was fermented for each forage species and method of sample preparation at different times of incubation is given in Figure 4-5.

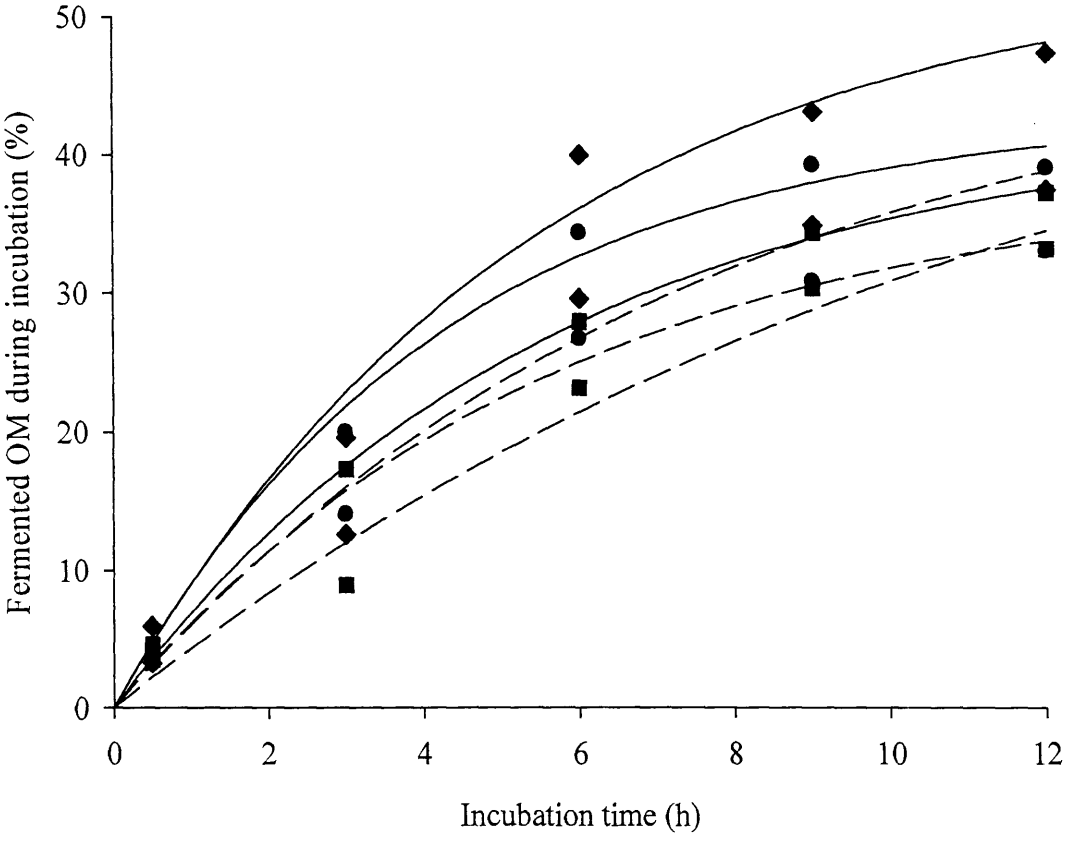


Figure 4-5. Cumulative OM fermentation of lucerne (—) and ryegrass (---) incubated fresh (♦), frozen-thawed (■) or frozen-thawed + starch (●) at different incubation sampling times.

There was a significant ($P<0.01$) difference in mean predicted OM fermentation between forage species (L 41.2 *v.* R 34.2% ± SEM 0.29). Mean of fermented OM was significantly ($P<0.01$) higher for fresh forage samples compared to frozen-thawed and frozen-thawed + starch samples (42.4 *v.* 35.2 *v.* 36.0% ± SEM 0.35). As incubation time increased the amount of OM fermented increased

significantly (P<0.01). There were significant (P<0.01) interactions between forage type and preparation method, forage type and incubation time (P<0.01) and incubation time and preparation method (P<0.01). However, there was no significant (P=0.32) three-way interaction between S, PM and T for OM fermentation. Also, Figure 4-5 shows that regardless of preparation method, lucerne OM was fermented more quickly than ryegrass.

4.3.3 Gas production

The patterns of cumulative gas production (corrected for production by blank at each incubation time) between forage species, preparation method (PM) and incubation times (T) are shown in Appendix 2. The quantity of gas produced was higher in lucerne than ryegrass. The effect of method of sample preparation on gas production quantity was fresh> frozen-thawed plus starch> frozen-thawed.

4.3.4 Rumen stoichiometry

The quantity of products during 12 h incubation *in vitro* of forages prepared by three different methods is given in Table 4-4. Fresh samples produced significantly (P<0.01) higher amounts of fermentable glucose, ATP, TVFA and MCP than samples prepared from frozen forage. However, there were highly significant differences (P<0.01) between forage species and preparation method except for gas production which differed (P<0.05) among preparation method and did not differ between forage species (P>0.05).

Table 4-4. Effect of forage type and method of sample preparation on the quantity of products formed after a 12 h incubation of 1 g OM by rumen microbes calculated by stoichiometric predictions from measured TVFA production

	Lucerne			Ryegrass				*P values*		
	F	FT	FTS	F	FT	FTS	SEM	S	P	S*P
Observed										
TVFA (mmol/g OM)	5.27^{d}	4.23^{b}	4.40^{c}	4.25^{b}	3.81^{a}	3.73^{ab}	0.13	<0.01	<0.01	<0.01
Gas (mmol/g OM)	27.2^{d}	16.5^{ab}	21.4^{b}	23.2^{b}	16.0^{a}	21.3^{c}	1.27	NS	<0.05	NS
Calculated										
Glucose (mmol/g OM)	2.96^{d}	2.32^{b}	2.44^{c}	2.34^{b}	2.07^{a}	2.06^{ab}	0.08	<0.01	<0.01	<0.01
ATP (mmol/g OM)	11.9^{d}	9.46^{b}	9.90^{c}	9.58^{b}	8.42^{a}	8.54^{ab}	0.31	<0.01	<0.01	<0.01
MCP (mg DM)	119^{d}	94.6^{b}	99.0^{c}	95.8^{b}	84.2^{a}	85.4^{ab}	3.08	<0.01	<0.01	<0.01
Gas (mmol/g OM)	35.4^{bc}	27.6^{b}	31.0^{bc}	26.8^{a}	23.2^{a}	24.6^{a}	0.61	<0.01	<0.01	<0.01

Means within a row without a common superscript differ (P<0.05)

4.3.5 Recovery of N as ammonia and MCP

An internal plant N marker (^{15}N) was used in this study to identify N released from the plant materials during the incubations. The cumulative recoveries over time of N in ammonia (%) from the total N originally in both forage species, prepared for incubation by three methods are given in Figure 4-6. Overall, mean cumulative N recovery increased significantly ($P<0.01$) as incubation time increased. The recoveries (%) differed ($P<0.01$) between species (L 47.2 *v.* R 55.9% ± SEM 1.05) and due to PM (F 53.9 *v.* FT 48.2 *v.* FTS 52.6% ± SEM 1.28) ($P<0.01$). There were significant interactions between S and PM ($P<0.01$), S and T ($P=0.01$) and PM and T ($P=0.05$). There was a significant ($P=0.01$) three way interaction between S, PM and T.

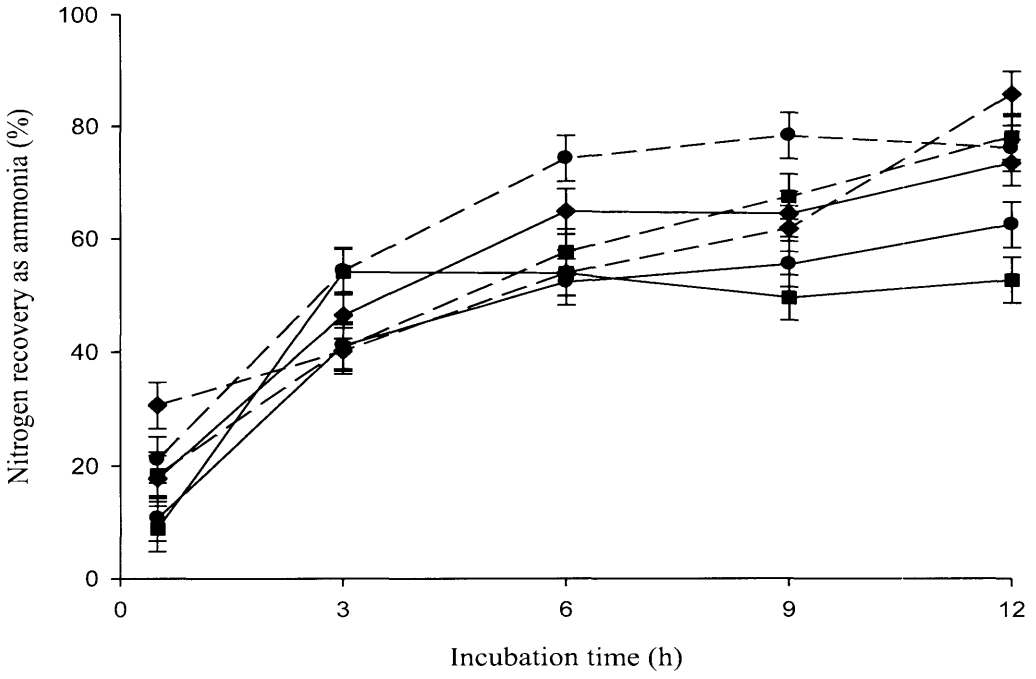


Figure 4-6. Feed N recovery as ammonia-N (%) from lucerne (—) and ryegrass (---) for each of 3 sample preparation methods, i.e. fresh (♦), frozen-thawed (■) and frozen-thawed + starch (●) at different incubation sampling times (Mean ± SEM).

The recovery of ^{15}N from labelled plant material in ammonia (%) is presented in Figure 4-7.

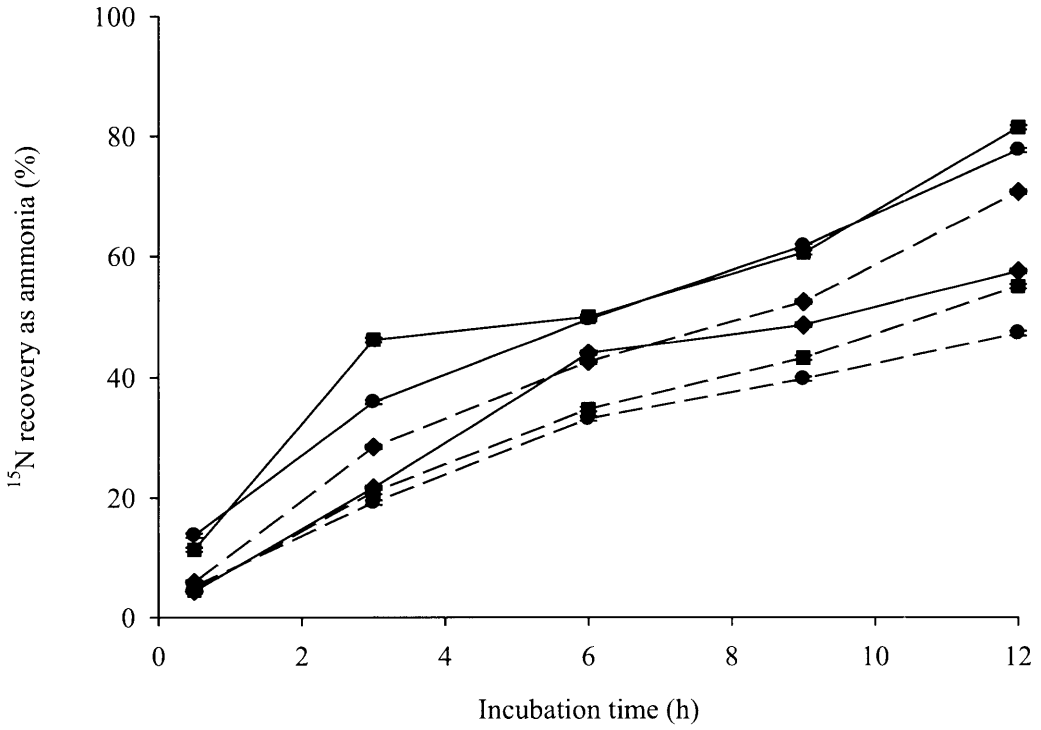


Figure 4-7. ^{15}N recovery as ammonia (%) from lucerne (—) and ryegrass (---) for each of the sample preparation methods, i.e. fresh (♦), frozen-thawed (■) and frozen-thawed + starch (●), at different incubation times (Means ± SEM).

An estimate of the ^{15}N recovered as ^{15}N ammonia in the incubation bottle (see Figure 4-7) was given by multiplying the concentration of ammonia produced from forage incubation by its ^{15}N enrichment. Recovery of ^{15}N in MCP was not measured in this experiment. The mean percentage recovery of forage N in MCP calculated from stoichiometry was significantly ($P<0.01$) lower for lucerne than ryegrass (L 13.1 v. R 14.8% ± SEM 0.15). Method of sample preparation had a significant ($P<0.01$) effect on N recovery in MCP (F 14.2 *v.* FT 11.3 *v.* FTS 16.3% ± SEM 0.19). Significant interactions were found between forage type and method of sample

preparation (P=0.01). Total recovery of forage N in NH_3 plus MCP is presented in Figure 4-8.

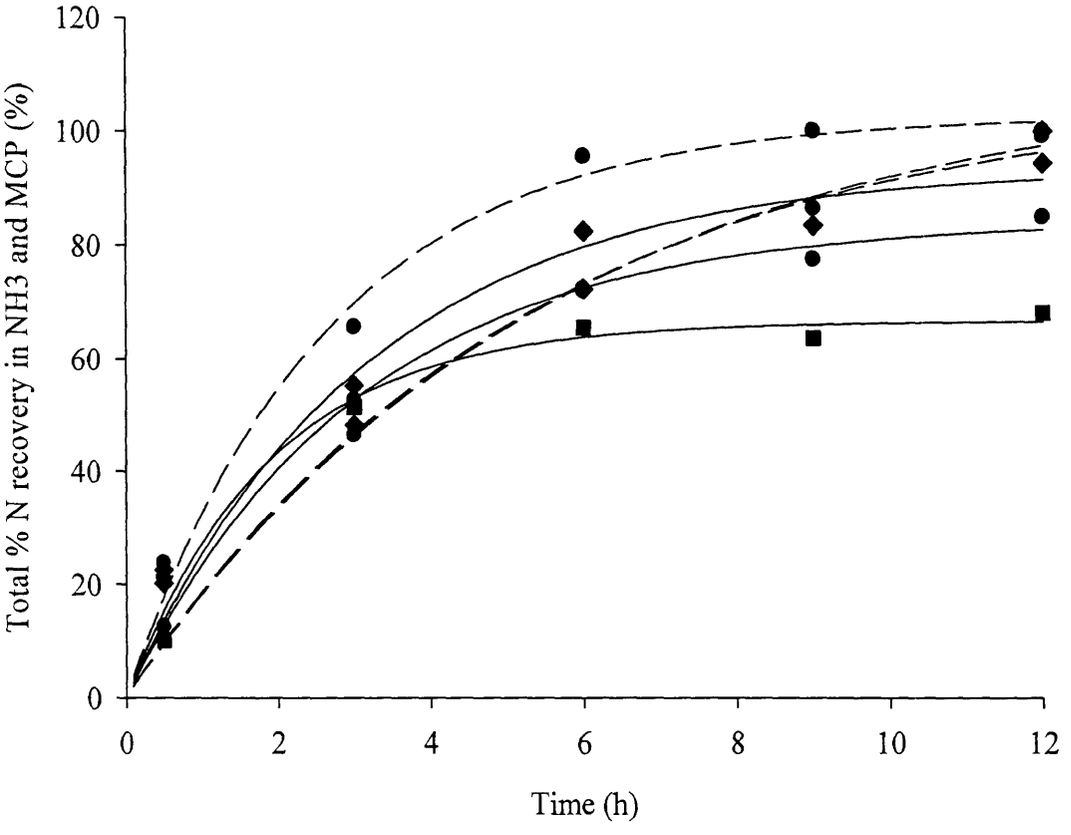


Figure 4-8. Total recovery of forage N in NH_3 and MCP (%) in lucerne (—) and ryegrass (---) for each of the sample preparation methods, i.e. fresh (♦), frozen-thawed (■) and frozen-thawed + starch (●), at different incubation times

Curves were fitted to the cumulative recoveries of OM fermented (Figure 4-5), total VFA production (Figure 4-3) and recovery of N as NH_3 and MCP (Figure 4-8) and the coefficients are given in Table 4-5.

Table 4-5. Coefficients of fitted curves of TVFA (mmol g/OM)), total recovery of N (Total N %) and percentage of OM in the incubation flask that was fermented (FOM)

		Lucerne			Ryegrass				*P values*		
	Coefficients	F	FT	FTS	F	FT	FTS	SEM	S	P	S*P
TVFA											
	a	6.07^{b}	4.91^{a}	4.84^{a}	5.60^{b}	6.34^{b}	4.32^{a}	0.17	NS	<0.01	<0.01
	b	0.18^{b}	0.17^{b}	0.23^{c}	0.13^{ab}	0.08^{a}	0.17^{b}	0.02	<0.01	<0.01	NS
Total N											
	a	94.7^{b}	66.7^{a}	85.0^{ab}	133^{bc}	112^{b}	107^{b}	7.75	<0.01	<0.05	NS
	b	0.30^{a}	0.78^{b}	0.32^{a}	0.14^{a}	0.17^{a}	0.34^{a}	0.09	<0.02	NS	<0.05
OM											
	a	54.3^{b}	43.3^{a}	43.2^{a}	48.8^{b}	55.0^{b}	38.4^{a}	1.42	NS	<0.01	<0.01
	b	0.18^{b}	0.18^{b}	0.23^{bc}	0.13^{ab}	0.08^{a}	0.18^{b}	0.02	<0.01	<0.01	NS

Means within a row without a common superscript differ (P<0.05)

a is the soluble fraction or easily degradable and *b* is the fraction potentially degradable.

In Table 4-5, total N recovery was significantly different between species and it was greater in ryegrass than lucerne but generally lucerne were degraded quicker than ryegrass. Method of sample preparation were also significantly different (except for *b* value of Total N) between for all the coefficients of fitted curves. .

4.4 Discussion

4.4.1 Incubation characteristics

For *in vitro* incubations, it is recommended to use rumen fluid collected after overnight fasting because microbes are less active (Williams 2000). A mixture of the rumen fluid from at least two donor animals is also recommended as this promotes a greater constancy of activity (Menke *et al.* 1979; Menke and Steingass 1988). The rumen fluid used in this experiment was taken from four Merino sheep which made us more confident that there would be a range of rumen microflora present. However, there was still unfermented nitrogenous material in the rumen fluid as ammonia production continued in the blank flasks during the 12 h incubation period. The pH of rumen fluid in blank samples was almost constant after 12 h however; adding a slurry of fresh or frozen-thawed forage increased the initial pH of the medium but as the incubation progressed it was decreased because of the production of VFA and gases. The capacity of the McDougall's buffer is an important factor for neutralizing the acidity of the VFAs produced during the incubation and preventing fluctuations of pH. A secondary role of the buffer is to supply essential minerals for optimal microbial activity. The buffer appears to have achieved both these roles in this experiment.

4.4.2 Fermentation

4.4.2.1 Volatile fatty acids

Tahmasbi *et al.* (2005) processed fresh kikuyu (*Pennisetum clandestinum*) in three different ways before incubating the samples *in vitro* and determining the

VFA profiles. They concluded that, when *in vitro* tests are used for assessing fermentability of forages, the method of sample preparation can affect the concentrations of VFA produced and therefore also the conclusions drawn. The results obtained in this experiment with lucerne and the results with ryegrass support the results with kikuyu showing that method of sample preparation can affect the fermentation characteristics of forage samples.

Boss and Bowman (1996) found that the quantity of VFA produced corresponds to the amount of fermentable OM ingested and the ratio of acetate to propionate (C2:C3) produced is lower, as more propionate is being produced when the rate of fermentation is higher. In this experiment, the ratio of C2:C3 was higher for frozen-thawed than other treatments, so it was predictable that the fermentation rate would be lower in stored than in fresh samples. This theory was supported by the predicted rates of fermentation of OM which were higher when fresh material was being fermented. Adding starch to the incubation vessels containing lucerne increased the quantity of propionate produced relative to acetate (lower C2:C3), suggesting that the starch was rapidly fermented, but the rates of TVFA and the ratio of C3:C2 were still generally lower than when the same amount of fresh sample was incubated.

Fresh forage samples produced significantly ($P<0.01$) more VFA than frozen-thawed samples throughout the 12 h incubations. It seems that the fresh forage samples had more available sugar than frozen-thawed samples and that even the non-starch OM may have been more rapidly and more completely degraded. On the other hand, Pollock (1984), cited by Tabaei Aghdaei *et al.* (2003) suggests that at the beginning of exposure to cold conditions, plants accumulate soluble carbohydrates in preparation for the cold situation and that soluble carbohydrate content of grasses can increase up to 10 times within 8 h of transfer from a warm to a cold environment. Apparently, soluble carbohydrate production did not occur in the frozen forage in this study as more fermentation would have been expected to occur when the samples that were frozen before storage were incubated, and this was the opposite of what occurred. Perhaps there was a loss of carbohydrate during the final stages of cooling or during the thawing process. However, MacRae (1970) suggested

that, if frozen-thawed grass is consumed by feeding animals within 12 h after taking it out of the deep freezer, the risk of loss of soluble carbohydrate components during thawing is avoided. With this range of possibilities, and the knowledge that the fermentation of the frozen-thawed material increased when starch was added, it is difficult to predict what changes occurred in the fermentable carbohydrate concentrations in the samples during freezing and thawing in this study. This increases uncertainty about evaluations of fresh forages *in vitro* using frozen-thawed samples.

4.4.2.2 Gas production

Volatile fatty acids and gases (CH_4 and CO_2) are the end-products of OM fermentation in the rumen. However, some CO_2 can also be released from the buffer when pH is reduced by VFA production (Menke *et al.* 1979). In this experiment there was a linear relationship between *in vitro* gas production and VFA production, as has also been found by other researchers (Blummel *et al.* 1997; Yang *et al.* 2005).

Incubations of fresh samples produced more gas and reached maximum fermentation rate more quickly than those from incubation of frozen-thawed samples. This was probably due to the presence of more readily fermentable carbohydrate and also a more rapid colonization of fresh samples by rumen micro-organisms compared with the frozen-thawed samples. By adding starch to the frozen-thawed samples, the quantity of produced gas was increased, but gas production was still less than for fresh forage. Added starch provided additional readily fermentable substrate for the microbial populations but the fermentable OM present was still lower than in fresh forage.

In this study it was found that there was a close relationship ($r^2 = 0.92$) between calculated and observed gas production rates for all treatments, although the mean calculated values were higher by about 30% than mean of observed ones, which is close to the value reported by Beuvink and Spoelstra (1992). Opatpatanakit *et al.* (1994) observed a close relationship ($r^2 = 0.94$) between a variety of starches in cereals, but in their experiment the expected production rates were slightly greater

than the observed values. Blummel and Ørskov (1993) also reported a high correlation between expected and observed volumes with forages, but the correlation was closer at 24 than at 48 h. Groot *et al.* (1998) reported that the measured gas volume when cell contents of Italian ryegrass were incubated *in vitro* was noticeably lower than that predicted from VFA produced, especially during the first 24 h of incubation.

Acetogenesis may be more extensive in species other than ruminants, especially Australian marsupials (Ouwerkerk *et al.* 2007) but, in ruminants, occurs mainly when low roughage diets (25% NE_m from alfalfa and 75% NE_m from a concentrate mix (as fed percentage)) containing high proportions of sugar and protein are fed (Leedle and Greening 1988). Another reason why actual production was overestimated might be the high CP content of the forages. Cone and Van Gelder (1999) compared gas-production profiles of several feed samples and suggested that the lower gas production associated with fermentation of protein could be explained by the inhibiting effects of ammonia on gas release from the buffer. Also, Schofiled (2000) reported that ammonia in high protein feeds can decrease gas production by reaction with volatile fatty acids.

4.4.3 N recovery as ammonia and MCP

Satter and Slyter (1974) indicated that the minimum ammonia-N concentration for optimal microbial protein synthesis *in vitro* is about 50 mg N/L. The concentration of ammonia-N *in vitro* is a balance between rates of ammonia production by forage degradation and assimilation by micro-organisms from the incubation media. Because *in vitro* incubation takes place in a closed system, deamination of amino acids by the bacterial population and secretion of small amounts of ammonia from protozoa are the main sources of ammonia production (Hsu *et al.* 1991b). All of the ammonia-N removed from the incubation environment is by microbial assimilation for their biomass synthesis and so the situation differs from that *in vivo* where ammonia is also removed by absorption across the rumen wall and by outflow in digesta. The concentration of ammonia-N in the medium will therefore probably exceed that if the same forage is ingested by an animal and, in this

in vitro experiment, the concentrations achieved at the end of the 12 h incubation exceeded 500 mg N/L. Concentrations were therefore higher than those normally found *in vivo* and would certainly not have been limiting for microbial fermentation and growth.

In the present experiment, the recovery of N in ammonia was lower during the incubation of frozen-thawed than for fresh forages. When an energy source (1 g starch) was added, N recovery increased but was still lower than for fresh forage. However, regardless of forage type, when energy sources were added to the incubation system, microbial protein synthesis increased, again confirming that the availability of ammonia-N for microbial protein synthesis was non-limiting for microbial growth. Adding supplemental starch or increasing dietary starch digestibility has been shown to alter ruminal fermentation (Poore *et al.* 1993). There is also evidence that cellulose digestion is reduced when large amounts of readily available carbohydrates are available (Stern *et al.* 1978).

As shown in Figure 4-8, the percentage of recovery of N from ryegrass appeared to be greater than 100% and the percentage recovery of N from lucerne was generally lower than from ryegrass. The likely reason is that lucerne had a higher crude protein concentration than ryegrass and so more N was present in the flask at the start of incubation. Proportionally less N was therefore used for microbial protein synthesis. Plant breeders have responded to the low ruminal recoveries of N in ryegrass by selecting for high-sugar varieties in an attempt to more closely balance the N and carbohydrate concentration in the rumen (Aikman *et al.* 2002; Miller *et al.* 2001). The results from the present study suggest that, for ruminant feeding, plant breeders should select varieties of lucerne with higher levels of soluble sugars or, alternatively for lower N concentrations or for nitrogenous materials that is more likely to escape fermentation in the rumen.

Preparation of the forages after cutting is an important factor in determining the rumen degradation of forages. In their *in vivo* experiment, Beever *et al.* (1974a) reported that net loss of N from the rumen was higher for fresh than frozen-thawed ryegrass. They also concluded that freezing did not have any significant effect on apparent N absorption from the small intestine. MacRae (1975) reported that freezing

caused a reduction in N solubility due to vacuolar membrane breakdown which can markedly boost the potential microbial utilization of the dietary protein by ruminants.

The *in vitro* method was able to identify differences in the fermentation patterns and degradability parameters for fresh lucerne and ryegrass; therefore provided useful information about their potential nutritive value as grazed forages. However, freezing and thawing the forage samples affected their fermentation and microbial growth characteristics. Freezing and storing samples before evaluating them by the *in vitro* method could therefore lead to false conclusions about their nutritive value for grazing animals.

Chapter 5 The degradation of fresh and frozen ^{15}N-labelled lucerne and ryegrass determined by the *in situ* technique

5.1 Introduction

In the previous chapter, the *in vitro* technique was used to determine the nutritive value of fresh forages as sources of N and fermentable energy for ruminants. Incubation of feed in porous nylon bags in the rumen is another commonly used technique that takes advantage of the real rumen environment to estimate the degradation characteristics of different feedstuffs (Hoffman *et al.* 1993; Kempton 1980; Orskov 2000). Rumen micro-organisms can enter the bags, attach to, and ferment and degrade the feed and this enables rates of disappearance of DM and N and effective degradability to be determined. A disadvantage of the procedure is that microbial cells contaminate the feed residues so that DM and especially CP degradation are under-estimated.

When determining the degradability characteristics of fresh forages available to grazing livestock the manner in which samples are prepared in order to simulate the *in vivo* ingestion process before incubation can also affect degradability estimates. For fresh forages, cutting the material into small pieces before incubating

in the rumen is needed to simulate the effects of chewing by the animal; however, crushing is a key part of bolus formation alongside cutting . Methods of preparation of herbage samples include oven or freeze drying, chopping, grinding and mincing; the use of masticated preparations are less common.. Preparation of fresh samples should be done as soon as possible to avoid wilting and/or the loss of soluble fractions. Often the testing of fresh samples is difficult because it is necessary to have access to the animal and the forage samples in the same place and at the same time. Therefore, samples are commonly preserved by freezing or drying and are incubated later in the rumen. The changes that occur during the preservation and storage processes could mean that the results are not representative of the original fresh forage material.

This study was undertaken to compare the degradation of DM and N *in situ* of two species of forages (lucerne and ryegrass) when incubated in the rumen as fresh or frozen and thawed material. The forages were labelled with ^{15}N which enabled the disappearance of plant N from the bag to be monitored and the extent of microbial contamination of feed residues in the bag and resulting under-estimation of effective feed N degradation to be calculated.

5.2 Material and Methods

The experimental design was a 2×2 factorial (2 forage species, 2 preparation methods) and each treatment was replicated in 3 sheep.

5.2.1 Animal and basal diets

The samples for each of the treatments were incubated in 3 rumen-cannulated sheep kept in individual pens in an animal house at the University of New England. They had free access to drinking water and received a basal diet of chopped lucerne hay (600 g/d, 92% DM, 19% CP) and oaten chaff (400 g/d, 91.5% DM, 7.5% CP) daily at about 0900 h.

5.2.2 Sample preparation and incubation

Two forage species, lucerne (*Medicago sativa* L.) and ryegrass (*Lolium perenne* L.) were used in this study. Both forages were labelled with ^{15}N during their growth in pots as described in Section 4.2.1. The lucerne was harvested before blooming stage (second cut, 22% DM, 22.7% CP), and either prepared as fresh or frozen-thawed material. Fresh samples were incubated on the day of harvesting or, for the frozen-thawed treatment, were immediately transferred to a deep freezer at -20 °C, kept frozen in sealed plastic bags and thawed and placed in the rumen of the sheep 2 days later. The same procedure was applied to third-cut ryegrass (17% DM, 13.7% CP). Details of treatments and incubation days are shown in Table 5-1.

Table 5-1. Treatments and two-day incubation periods

Forage species (S)	Method of sample preparation (P)	Incubation day
Lucerne	Fresh (F)	1-2
	Frozen-thawed (FT)	3-4
Ryegrass	Fresh (F)	5-6
	Frozen-thawed (FT)	7-8

The period between cutting growing plants in the glass house and placing the prepared samples in bags in the rumen was less than 30 min. The frozen samples were taken out of the deep freezer (-20 °C), thawed at room temperature for 30 min and weighed as for fresh samples. There was no leakage of fluid from the thawed material of either species. Fresh or FT lucerne or ryegrass (about 1 g DM) was chopped by scissors to approximately 0.5 cm lengths and added to 24 pre-weighed polyester bags (7 cm x 4 cm, pore size of 44 μm x 44 μm; Swiss Screen, Seven Hills, NSW 2147). A marble was put into each bag to ensure that the bag remained in the liquid phase of the rumen contents during incubation and to facilitate its removal from the rumen when the desired incubation time had concluded. The bags were then re-weighed and tied to the end of a 100-cm fishing line (20 lb / 9 kg). Seven bags were chosen at random and placed in the rumen of each of the 3 sheep as replicates at

the same time. Three bags were chosen at random to be 'zero time' samples. The 7 bags in the rumen were removed successively after 1, 3, 6, 9, 12, 24 and 48 h of incubation. The protocol for the measurement of *in situ* degradability of samples was similar to that recommended by the Agricultural and Feed Research Council (AFRC 1992), the only difference being that the sheep were offered feed once-daily.

Immediately after removal from the rumen, the bags were put into a bucket of cold water, feed particles were washed off by hand, and each bag was then rinsed separately under running cold tap water for about 2 min. The bags and their contents were then transferred to another bucket and cleaned under running cold tap water to ensure coarse particles trapped around the mouth of the bag were removed. Bags that were not incubated in the rumen were washed and dried as a similar manner. The washed bags were gently squeezed, placed in an aluminum tray and dried in a forced-draught oven at 65°C for 48 h. The dried bags and contents were cooled in a desiccator, weighed (for DM calculation) and stored pending N and ^{15}N analysis.

5.2.3 Chemical analysis

N concentrations in bag residues were determined on the dry samples after they had been ground to pass a 1 mm sieve (Foss Tecator, 1093 cyclotec sample mill). Concentrations of N and ^{15}N in the samples were determined as described in Section 3.2.8.

5.2.4 Calculations

Rumen degradability parameters were calculated as the percentage of disappearance of DM, N and ^{15}N from the bags at different times during incubation and fitted by the following equation (Orskov and McDonald 1979):

$$P_t = a + b(1 - \exp^{-ct}) \qquad (8)$$

where P_t indicates the percentage of the material removed from the bag at time t; a is the soluble potentially degradable fraction; b is the insoluble potentially

degradable fraction; *c* is the degradation rate constant for fraction *b*. The percentage disappearance with time values were fitted by using the 'Neway' program for Microsoft Excel (Chen 1997).

Effective rumen degradabilities of DM and protein were calculated using the fractional outflow rate of solid from the rumen (k_p) according to the following equation.

$$P = a + ((bc)/(c + k_p))(1 - e^{-ct}) \qquad (9)$$

where 'p' is the effective extent of degradation at time t(h), k_p is the particle flow rate constant assumed to be 0.05/h (Hristov and Broderick 1996). *a*, *b* and *c* are the same as mentioned in equation $Deg(t) = a + b[c/(c+k)]$ (18.

The rumen microbial N (RMN^{15}_N) in the feed residues derived from ^{15}N values, was calculated using the equation given by Varvikko and Lindberg (1985).

$$\left(1 - \frac{^{15}N\%\ of\ total\ residual\ N}{^{15}N\%\ of\ total\ original\ N}\right) \times 100 \qquad (10)$$

The percentage of error in the feed N loss estimates resulting from the microbial contamination of the feed sample residues was calculated from the following equation.

$$\left(1 - \frac{\%\ ^{15}N\ disappearance}{\%\ Total\ N\ disappearance}\right) \times 100 \qquad (11)$$

5.2.5 Statistical analysis

Data were analyzed as a 2×3 factorial design using repeated measures with the MIXED procedure of SAS v. 9.1.3 (SAS 2003):

$$Y_{ijk} = \mu + S_i + P_j + (SP)_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ= overall mean; S_i = main effect of forage species P_j =main effect of preparation method; $(SP)_{ij}$= average effect of interaction of forage species *i* and preparation method *j*; ε_{ijk}= residual error

5.3 Results

Mean degradabilities of fresh and frozen-thawed lucerne and ryegrass are presented (Figure 5-1 and Figure 5-2). The degradation parameters from the equation (8) are presented in Table 5-2.

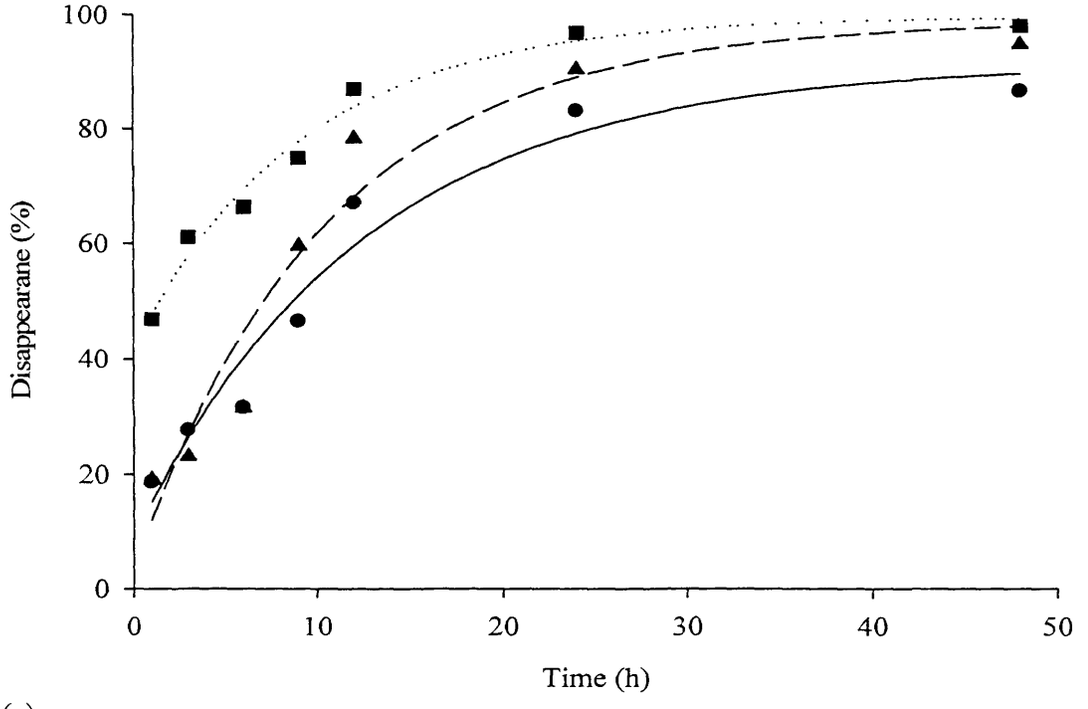
The soluble fraction *a* of DM and N was significantly ($P<0.001$) higher in frozen-thawed than fresh forages. The corresponding values for the potentially degradable *b* of N also differed for DM ($P<0.01$) and N ($P<0.05$). The degradation rate constants *c* differed for DM ($P<0.01$), N ($P<0.01$) and ^{15}N ($P<0.05$). The interactions between forage species and preparation method were only significant for degradation parameters of DM and ^{15}N washing loss ($P<0.05$).

The DM washing loss was significantly ($P<0.001$) higher in ryegrass than lucerne but no significant difference was achieved for N and ^{15}N due to sample preparation. However, there were significant differences between forage species in washing loss of N ($P<0.05$) and ^{15}N ($P<0.01$) and these differences were highr in lucerne than ryegrass. The potential degradability of DM differed between forage species ($P<0.05$) and sample preparation method ($P<0.01$). The effective degradabilities of DM ($P<0.01$), N ($P<0.01$) and ^{15}N ($P<0.05$) were significantly higher in lucerne than ryegrass.

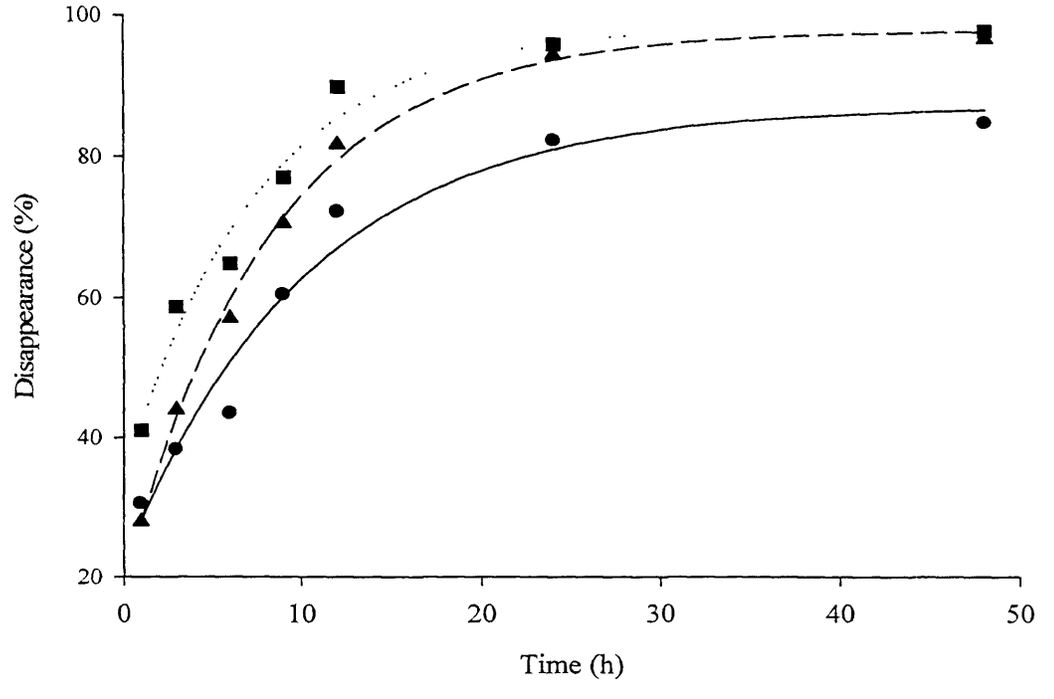
Table 5-2. *In situ* degradation parameters of DM, N and ^{15}N for fresh and frozen-thawed lucerne and ryegrass and their effective degradabilities at an assumed rumen outflow rate of 5%/h.

Processing method (P)	Forage species (S)					*P* values		
	Lucerne		Ryegrass					
	Fresh	Frozen-thawed	Fresh	Frozen-thawed	SEM	S	P	S*P
Degradability								
DM								
a	9.4^{e}	21.8^{d}	11.9^{e}	20.1^{d}	1.37	NS	<0.01	NS
b	84.3^{d}	65.4^{e}	82.7^{d}	77.4^{d}	2.72	NS	<0.05	<0.05
c	0.07^{e}	0.09^{d}	0.05^{de}	0.04^{f}	0.01	<0.01	NS	<0.05
WL (%)	3.1^{e}	16.6^{d}	3.5^{e}	14.4^{d}	1.87	NS	<0.01	NS
PD (%)	93.7^{d}	87.3^{e}	94.7^{d}	93.2^{d}	1.35	<0.05	<0.05	NS
ED(%)	59.9^{de}	65.1^{d}	56.1^{f}	58.4^{f}	1.70	<0.05	NS	NS
N								
a	3.4^{f}	18.7^{d}	7.6^{ef}	13.8^{de}	2.06	NS	<0.01	NS
b	95.6^{d}	79.1^{e}	89.1^{d}	82.2^{e}	1.99	NS	<0.01	NS
c	0.09^{e}	0.12^{d}	0.07^{f}	0.08^{ef}	0.01	<0.01	<0.05	NS
WL (%)	16.1^{d}	16.3^{d}	11.7^{d}	12^{d}	2.40	<0.05	NS	NS
PD (%)	99.1^{d}	98^{d}	96.7^{d}	94^{d}	2.51	NS	NS	NS
ED(%)	66.3^{e}	74.8^{d}	59.7^{e}	62.2^{e}	2.42	<0.05	NS	NS
ECP (%)	33.7	25.2	40.3	37.8	2.41	<0.05	NS	<0.05
^{15}N								
a	43.7^{d}	33.7^{d}	14.2^{e}	15.4^{e}	3.21	<0.01	NS	NS
b	56.1^{e}	64.8^{e}	94.3^{d}	86.6^{d}	3.93	<0.01	NS	NS
c	0.11^{de}	0.13^{d}	0.06^{f}	0.08^{ef}	0.01	<0.05	NS	NS
WL (%)	23.2^{d}	27.5^{d}	16.3^{e}	9.4^{f}	1.95	<0.01	NS	<0.05
PD (%)	99^{d}	98.5^{d}	100^{d}	100^{d}	0.67	NS	NS	NS
ED(%)	81.6^{d}	80.7^{d}	63.8^{e}	67.4^{e}	2.02	<0.01	NS	NS

Means within a row without a common superscript differ (P<0.05), WL: washing loss; PD: potential degradability; ED: effective degradability, ECP: escape CP, (100 − ED)*(CP, g/kg of DM).

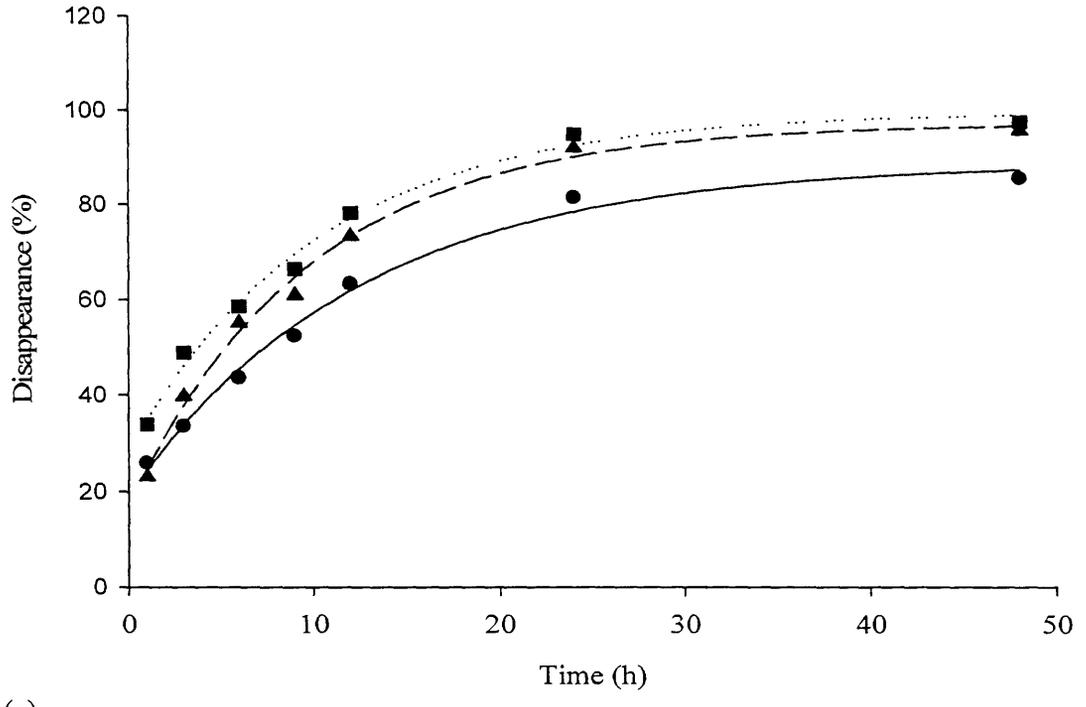


(a)

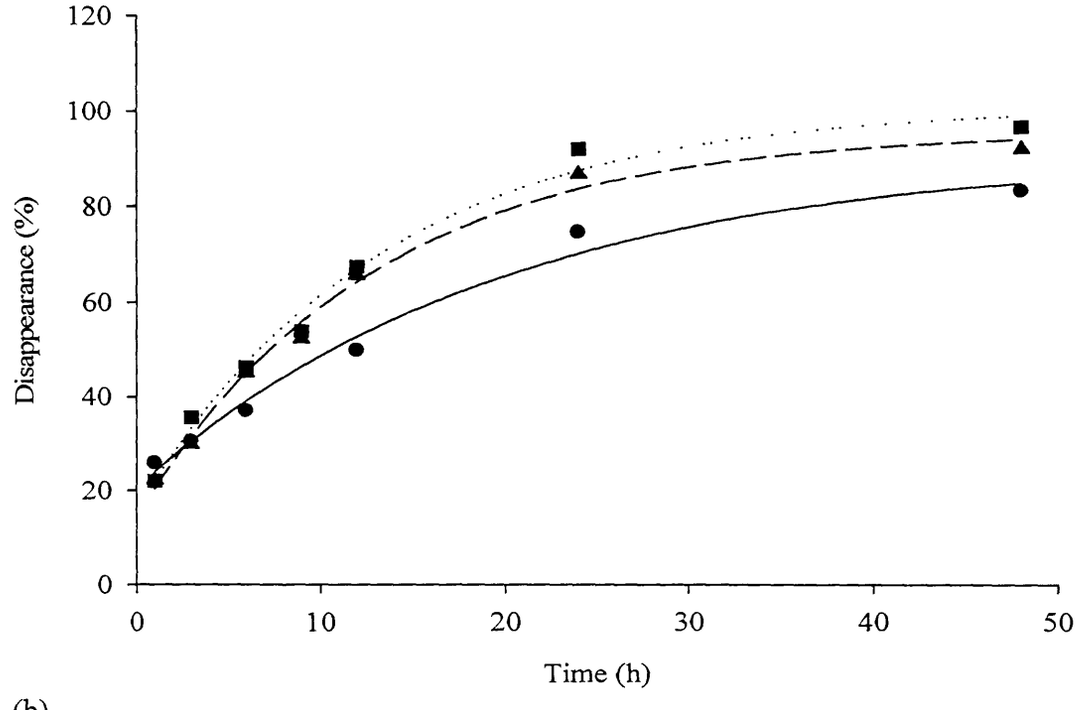


(b)

Figure 5-1. *In situ* disappearance (%) of DM (solid line), N (medium dash) and ^{15}N (dotted line) for fresh (a) and frozen-thawed lucerne (b)



(a)



(b)

Figure 5-2. *In situ* disappearance (%) of DM (solid line), N (medium dash) and ^{15}N (dotted line) for fresh (a) and frozen-thawed ryegrass (b)

The percentage of microbial N in the total N in samples throughout the period of incubation is shown in Figure 5-3.

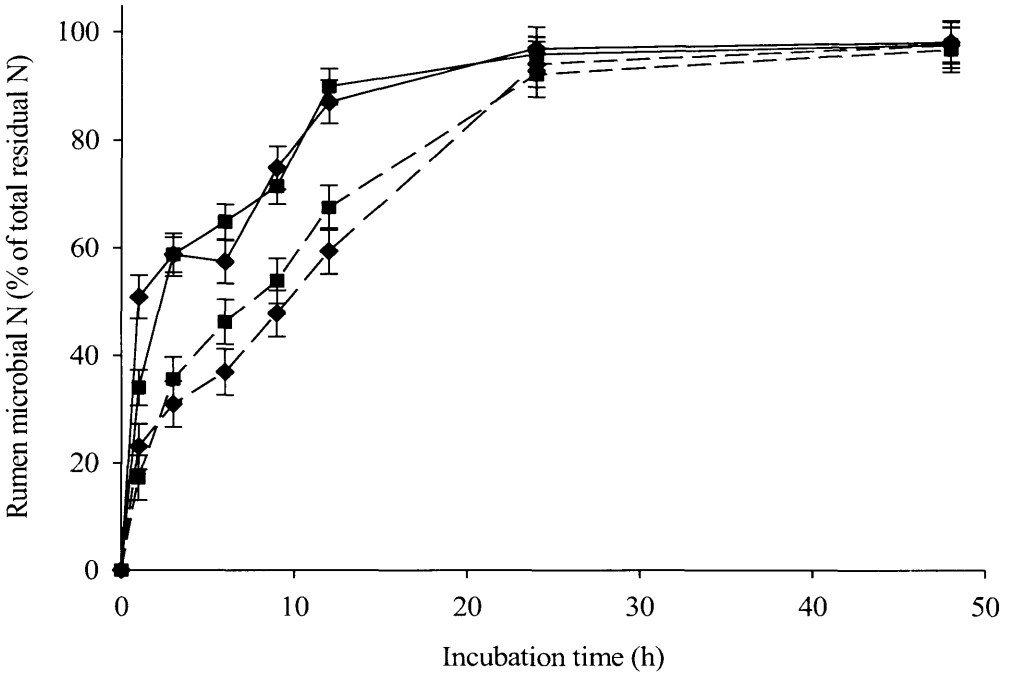


Figure 5-3. Mean ± SEM Rumen microbial contamination of fresh and frozen-thawed lucerne (—) and ryegrass (---) for each method of sample preparation: fresh (♦), frozen-thawed (■) at different sampling times (h) calculated according to (10.

As it is shown in Figure 5-3 there was a large amount of microbial contamination because of microbial attachment during the early hours of incubation, and was higher for lucerne than ryegrass. The percentage of error in estimation of forage N during *in situ* incubation was calculated from the values mentioned in Figure 5-3. The calculated errors in the forage N loss estimates from the microbial contamination of the feed sample residues left in the nylon bags are shown in Table 5-3.

Table 5-3. Error (%) in the forage N loss estimates as a result of microbial contamination of the feed sample residues

	Forage Species (S)						
	Lucerne		Ryegrass		*P* values		
Incubation time (h)	Fresh	Frozen-thawed	Fresh	Frozen-thawed	S	P	S*P
3	-25.7	-26.7	-22.3	-23.5	NS	NS	<0.0001
6	-17.5	-14.0	-14.7	-10.1	NS	NS	<0.0001
9	-12.6	-9.2	-10.4	-9.6	NS	NS	<0.0001
12	-11.1	-10.1	-5.0	-4.8	NS	NS	<0.0001
24	-7.1	-3.1	-3.9	-6.0	NS	NS	<0.0001
48	-3.4	-1.1	-1.9	-5.0	NS	NS	<0.0001

Means within a row without a common superscript differ (P<0.05)

The calculated error of forage N loss estimated from the microbial contamination was higher during the early hours of incubation, which corresponds to the values shown in Figure 5-3. Although contamination percentage was higher at longer incubation times, there was a low level of N in these residues so its effect on degradability estimates was small.

5.4 Discussion

Differences in the degradability characteristics of the two forage species, one a legume (lucerne) and the other a grass (ryegrass) were identified. There were also important differences in the degradability of DM and N as a result of the type of storage and preparation of the plant material. Moreover, the degradability values obtained by the *in situ* method were under-estimates because there was an error associated with the growth of, and attachment of, micro-organisms on the plant material in the bag as the incubation proceeds. An internal marker (^{15}N) in the plant materials was used in this study to estimate the true disappearance of plant N from the bags and to correct for microbial contamination of the plant material in the bags.

5.4.1 Forage sample size and preparation

During ingestion of fresh forage by ruminants, there is considerable particle size reduction by mastication (Ulyatt *et al.* 1986) and about 60% of the cell contents of fresh herbages are released (Waghorn *et al.* 1989). Any type of sample preparation is likely to inadequately represent the processes of ingestion, formation of a bolus and swallowing. AFRC (1992) recommended that, for *in situ* estimation of CP degradation in grass and silage, samples should be chopped to lengths of about 1 cm. However, in this experiment the forages were chopped to about 5 mm to better simulate the chewing activity of sheep.

Other workers have used different methods of sample preparation before samples are placed in the bags. Cohen and Doyle (2001) suggested that mincing fresh forage mimics the conditions occurring *in vivo*. They reported that CP solubility after chewing was 28-37% higher than the estimated values for chopped fresh white clover incubated *in situ*. Barrell *et al* (2000) reported that ranking of forages for digestibility was affected by method of preparation and they considered that mincing is preferable to either chopping or freeze drying, as particle sizes are closer to those present in real rumen contents. Deinum and Maassen (1994) investigated the effects of different methods of sample preparation. They proposed for example, mincing causes more damage to the plant cells structure or grinding would have increased the surface area of plant cells exposed for microbial attack compared with chopping. Currently, none of the methods of sample preparation has been nominated as the preferred method. It is likely that the most appropriate method for *in situ* studies will vary depending on whether fresh herbages or conserved forages and concentrates are being incubated (AFRC 1992).

5.4.2 DM degradation characteristics

Even though labelled lucerne and ryegrass were harvested at different stages of growth, there were no differences between fractions *a* and *b* for DM degradation among forages (see Table 5-2). Elizalde *et al.* (1999) showed that for lucerne and

ryegrass, the extent of ruminal DM degradation was affected by time of harvesting day, especially during later maturity.

In this study the effective degradabilities of DM and N were higher for lucerne than ryegrass. Waghorn *et al.* (1989) found that the residence time of lucerne in the rumen was shorter than for ryegrass, i.e. lucerne left the rumen more quickly than ryegrass. The higher effective degradabilities of DM and CP of fresh v. frozen-thawed forages in the rumen indicates there is more intensive microbial digestion of fresh material and that freezing has a disruptive effect on plant cell structure. Freezing and thawing significantly increased the soluble and effective degradable fraction of DM compared to fresh forages. Other studies have also shown that freezing increases rumen degradability of forages (Hristov 1998; Kamoun and Thewis 1990; Peyraud 1990). In this experiment the effect of the freezing tended to disappear, however, beyond 36 h of rumen incubation.

5.4.3 Nitrogen disappearance

The estimates of fractions *a* and *b* for N degradation were not different ($P>0.05$) between lucerne and ryegrass (see Table 5-2). The rate of CP degradation tended to be higher for lucerne than for ryegrass but there was no significant difference between the two forage species ($P = 0.41$). Compared to ryegrass, lucerne has a lower proportion of total N associated with NDF or ADF, and lower cell wall concentrations which may influence *in situ* N disappearance (Elizalde *et al.* 1999). The rate of CP degradation was related to the CP and NDF concentrations in the forage (Elizalde *et al.* 1999). They also concluded that rate of degradation of CP was higher in lucerne due to its higher CP and lower NDF concentrations than in ryegrasses.

Differences in rates of CP degradation between ryegrasses and lucerne in our experiment are in agreement with other experiments (Balde *et al.* 1993). The type of forage can also affect rumen *in situ* protein degradation. Vanzant *et al.* (1996) found greater effective degradability of prairie hay protein and lower effective degradability of lucerne protein when the forages were incubated in animals that

were ingesting brome hay than in animals ingesting the same forages that was being incubated. Wallace and Cotta (1988) indicated that the amount of dietary soluble protein could affect ruminal proteolytic activity, and Nugent *et al.* (1983) showed there is apparent competition for enzymatic sites between soluble proteins. Consequently, degradation characteristics of substrates incubated *in situ* can be affected by the quantity of soluble protein in the diet and the dietary proteins degradation rate (Vanzant *et al.* 1998). In this experiment, there was no significant difference between forage species in fractions *a* and *b* for N disappearance but the lower effective degradability of N in ryegrass relative to lucerne could be explained by both higher enzymatic activity of microbes on lucerne and lower degradation rate of N in ryegrass. This different enzymatic activity of microbes might be related to factors such as species composition, proteins structure, pH or ionic conditions.

High CP solubility affects degradability estimates made by the method of Ørskov and McDonald (1979). In this experiment, the highly soluble fraction (fraction *a*) in frozen-thawed samples was 3 times greater than for fresh samples. Similar variation was observed by Cohen and Doyle (2001). Consequently, interpreting protein degradability estimates of highly digestible fresh forages using certain preparations, particularly chopped material by the *in situ* technique needs more caution . Understanding the amount of rapidly released protein for rumen micro-organisms is important for optimizing rumen microbial protein synthesis and preventing inefficient utilisation of dietary protein because of high rates of ammonia production (Cohen 2001).

Wayman *et al.* (1995) found that the chemical composition and the microbial populations present on the grass *in vitro* were affected by freezing and thawing. They also concluded that there were differences in degradability characteristics of ensiled thawed grass compared with ensiled fresh grass, probably related to damage to plant cells and impaired microbial activity associated with freezing and thawing. Water extracts of frozen-thawed sample had higher concentrations of ammonia-N (NH_3-N) and total free amino acids (TFAA-N) than fresh lucerne. Frozen-thawed samples also had higher rumen DM and N solubility and higher effective degradability of DM and CP than fresh samples (Hristov 1998).

On the other hand, Dulphy *et al.* (1999) reported freezing of chopped fresh forage did not affect its N degradability *in situ*.

Even though there was no visible leakage of fluid in frozen-thawed sample in this study the water-soluble fraction of the total N from fresh forage samples was significantly smaller than that from frozen-thawed samples (3.4 *v.* 18.7%) and (7.6 *v.* 13.8%) in lucerne and ryegrass, respectively. The differences could be explained if there was disruption of cell membranes due to crystallization of intracellular content during freezing (Hristov 1998). The freezing and later thawing probably causes cell rupture and releases cell contents which contain soluble N and the water insoluble but potentially fermentable fraction in the rumen for frozen-thawed forages will be underestimated. During the first hours of freezing, water within the cell freezes forming ice crystals that penetrate the cell membrane. When the cell thaws, contents leak from the cells (Burke *et al.* 1976). However, Kohn and Allen (1992) did not find, microscopically, any changes in the surface morphology of frozen plant material.

There are few quantitative data on the amount of ruminal escaped crude protein (ECP) in fresh or direct cut lucerne. The values for proportion of total CP as ECP (Table 5-2) were, on average, 33.7 v. 40.3% in lucerne and ryegrass, respectively which is higher than the values reported by Elizalde *et al.* (1999). Their lower ECP estimates may have occurred because of the stage of maturity of fresh lucerne they used. The corresponding values for frozen-thawed forages were also higher in ryegrass than lucerne. Broderick *et al.* (1992) found, in freeze-dried lucerne incubated *in vitro*, an average of 24.0% of the CP escaped ruminal degradation, ranging from 33.6 (pre-bloom) to 17.4% (10% flowering).

Varvikko and Lindberg (1985) found calculation of microbial colonisation of feed residues in nylon bags using ^{15}N dilution might lead to an underestimate due to the re-use of the feed ^{15}N degraded inside the bags. In our experiment, during the first hour after incubation, the net disappearance of ^{15}N was greater than of total N. This result could be explained by the entry of rumen micro-organisms which have unlabelled N into the bag. Therefore, the presence of unlabelled N would lead to an under-estimation of true forage N degradability.

For both forages, the net disappearance rates ranked as follow: ^{15}N>N>DM. Because total N and DM fractions left in the bags were contaminated with micro-organisms, their rate of apparent or net loss can be expected to be slower than for ^{15}N, which is present only in plant N. The results of present study agree with the results of Varvikko and Lindberg (1985).

5.4.4 Microbial contamination

In situ incubation has been widely used for estimation of feed DM and protein degradation in the rumen but microbial colonization of feed particles in the bag leads to an underestimate of protein degradability by adding non-plant N to the residual N inside the bags (Mathers and Aitchinson 1981).

Several microbial markers have been used to quantify the extent of microbial contamination of feed residues. Mehrez and Orskov (1977) used diaminopimelic acid (DAPA) as a bacterial marker and reported microbial N contamination on barley residues was negligible. Varvikko and Lindberg (1985) used ^{15}N and DAPA as markers and found there was considerable feed contamination and concluded that estimating degradability of protein in forages and starchy feedstuffs by the *in situ* technique had significant source of error because of microbial colonisation on feed residues. Mathers and Aitchinson (1981) used ^{35}S as a marker, and concluded that lucerne and fish-meal residues were highly contaminated by microbes.

Measures of contamination of feed residues in this current experiment (Figure 5-1 and Figure 5-2) suggest that, as the period of incubation became longer, disappearance of ^{15}N from the bags decreased but the fraction present as microbial N increased. In addition, freezing increased the effective degradability of fresh lucerne and ryegrass CP. However, effective degradability of ^{15}N was higher in lucerne than ryegrass but was not affected by freezing.

The level of microbial contamination increases with increasing incubation time and has been shown to be greater in substrates with high fibre and low N concentrations (Beckers *et al.* 1995; Wanderley *et al.* 1993). Conversely, Mitchell *et*

al. (1997) found that with increasing plant maturity the protein residue was contaminated to a lower extent by rumen microbes, and they suggested that higher lignification may be associated with lower bacterial colonisation. The results in the current study suggest that forage species plays a minor role in the variation of forage CP degradable fractions and microbes tended to attach to lucerne more than to ryegrass. In contrast, Hoffman *et al.* (1993) found no relationship between extent of bacterial CP and maturity stage of forage species and correction for bacterial CP decreased the estimated undegraded CP fraction.

After 24 h incubation, forages residues were highly contaminated (up to 97%), which was in the same range as reported by Olubobokun *et al.* (1990) and Wanderley *et al.* (1993) but rate of attachment increased slowly between 24 and 48 h and calculated residual N contamination at 48 h in lucerne and ryegrass were 1.1 and 1.5 times greater than the amount at 12 h, respectively.

According to the results in Table 5-3 it seems clear that, in the nylon bag technique microbial attachment to the feed residues may be an important source of error in quantitative feed protein degradation. The degradation results also show that the extent of error (%) in the N loss measurement depends on the N content of the feeds and on the rumen degradation rate.

Because of the high quantity (about 97%) of microbial contamination achieved in this experiment on estimated N degradability, especially with fresh forages, it is necessary to consider microbial contamination in estimation of degradability parameters. Development of procedures for quick, precise and quantitative analysis of contamination deserves attention. Therefore, an *in vivo* experiment was conducted to obtain direct estimation of degradation rate of CP in fresh ryegrass when it is masticated and swallowed by sheep, and is reported in next chapter (see Chapter 6).

Chapter 6 Rumen degradation of fresh ^{15}N-labelled ryegrass after its ingestion by sheep

6.1 Introduction

The kinetics of the degradation of CP in fresh and frozen-thawed lucerne and ryegrass have been studied *in vitro* and *in situ* and reported in Chapters 4 and 5. There are important differences between the rumen environment *in vivo* and those *in vitro* or *in situ,* e.g. ammonia VFA and bicarbonate absorption through the rumen wall and outflow of digesta to the small intestine in digesta are not present *in vitro.* Moreover, the conditions in incubation vessels do not closely simulate those in the rumen (there are no rumen movements, or rumination cycles and there is no outflow of material) and so estimates of degradation rates are subject to considerable uncertainty. The *in situ* technique removes some of these limitations, but has other inherent problems, e.g. the preparation of material for incubation *in situ* may not truly represent material entering the rumen in the bolus after ingestion and there is no potential for the contents within the bag to be ruminated by the animal. Thus, the degree of chopping or masceration of the plant material before it is placed in the bags can be expected to affect the results. It is clearly more satisfactory to determine degradability values and degradation rates of DM and N *in vivo*, but the necessary experiments involve the use of experimental animals that incur costs in time and labor and raise animal welfare issues.

In studies *in vitro* and *in situ*, forages are cut to small pieces to simulate prehension, chewing, bolus formation and swallowing but the preparation is unlikely to closely simulate *in vivo* conditions. When ingesting fresh forages, animals masticate the plant material and form a bolus that is quickly swallowed. In grazing cattle, more than 50% of the cells are intact in relatively large particles when they enter the rumen and they may remain intact and alive for some time (Kingston-Smith and Theodorou 2000). Ingestive behavior can affect rates of release of cell constituents in the bolus. Boudon *et al.* (2006) for example, showed that intake rate of cows ingesting feeds indoors was higher (52 v. 23 g ○DM/min) and physical damage to plant material and release of intracellular N within the bolus was less than when they were grazing.

Quantitative models of N transactions in the rumen have been built using data obtained from *in vivo* experiments carried out using ^{15}N dilution techniques. In such experiments, ^{15}N-labelled ammonia is introduced into the rumen and the appearance of the ^{15}N label in secondary N pools such as microbial-N can then be determined. This type of tracer study can be a powerful means of identifying the sources of N used by micro-organisms in the rumen for their growth and protein synthesis. Nolan and Leng (1972), for example, reported that about 50% of microbial-N was derived from nitrogenous sources more complex than ammonia (*i.e.* non-protein NAN which includes peptides and amino acids) and about 50% was derived from ammonia when sheep were given lucerne diets. However, because ^{15}N-ammonia was the primary source of the ^{15}N tracer, the utilisation of plant non-protein NAN materials by micro-organisms could only be determined indirectly. Damry and Nolan (2002) used ^{15}N-labelled duckweed as the primary source of tracer and made direct estimates of non-protein NAN assimilation by rumen bacteria. Also, Hristov *et al.* (2001) investigated qualitative and quantitative aspects metabolism of N fractions in the rumen of cattle fed ^{15}N lucerne labelled as hay or as silage. They found ^{15}N enrichment of total N was higher in silage than hay, when both were prepared from the same lucerne plant. They also reported that when animals ingested lucerne hay, the ruminal non-protein, non-ammonia, non-microbial N (NPAM-N), consisting peptide-N pool comprised about 22% of total N in the rumen. This fraction was presumably mainly peptide and amino acid N.

Few of the ^{15}N studies with sheep or cattle so far undertaken have been made using fresh forage rather than preserved material. However, with sheep, Archimede *et al.* (1999) concluded that intake and digestibility of fresh pangola grass (*Digitaria decumbens*) was higher than that of the dried Pangola grass. Consequently, the rate of flow of nutrients in the digestive tract was higher for the animals fed fresh plant material. If this is generally true, measurements with dried grasses probably underestimate the nutritive value of the corresponding fresh ones. To evaluate the true nutritive value of pasture material, measurements of forage intake and digestibility must be made with fresh forage and the animal must be therefore allowed to masticate and swallow the forage.

In the study reported here and a separate study (using ^{15}N-labelled lucerne) (not reported in this thesis) the kinetics of protein degradation were determined for two different types of fresh forages, ryegrass and lucerne using ^{15}N dilution techniques. The N kinetics in the rumen were determined, as in many previous studies, by labelling the ruminal ammonia-N pool via intraruminal injection of $^{15}NH_4Cl$ and measuring ^{15}N enrichment over time in ruminal ammonia-N, bacterial N, peptide and amino acid N, and soluble protein N. In addition, the sheep were allowed to ingest a single meal of ^{15}N-labelled fresh ryegrass or ^{15}N-labelled lucerne were administered into the rumen and the transfers of the ^{15}N (i.e. plant N) to various N pools were determined.

In the experiments reported in this chapter, the objectives were to obtain direct information on the rate of degradation of CP in fresh ryegrass after it was ingested (masticated and swallowed) by sheep and to determine the rate of utilization of different degradation products for synthesis of bacterial protein or their rates of removal across the rumen wall or in digesta flowing out of the rumen.

6.2 Protein degradation of fresh ^{15}N-labelled ryegrass

6.2.1 Materials and methods

6.2.1.1 Planting and labelling fresh ryegrass

The ryegrass was grown in 4 L pots in a climate-controlled glass house (temperature and humidity were controlled). The soil used for planting was almost free of N (see Section 4.2.1 for soil composition) and therefore, at the time of planting, 8 g of Multigro fertilizer (N= 10.1%, P= 3.5%, K= 5.5%, S= 16.3%, Ca=7.8%) was added to the pots. Ryegrass plants were cut for the first time at 30 d after planting when they were at the four-leaf stage. On average 47.0 ± 1.44 g fresh ryegrass was harvested from each pot (DM % = 7.88 ± 0.08 g).

After the first harvest, pots were allocated at random to two groups; the first group was assigned to grow un-labelled ryegrass (control) and watered with tap water. The second group was assigned to grow labelled ryegrass. These pots were fertilized with a mixture of fertilizers including P, K, S, Ca (8.8, 50, 11 and 20%, respectively) and N was applied as $^{15}NH_4{}^{15}NO_3$. These pots (see Figure 6-1) were initially watered with $^{15}NH_4{}^{15}NO_3$ solution providing 30 mg ^{15}N and a further 12 mg ^{15}N was given to each pot weekly. The same quantity of un-labelled N was provided as fertilizer to the control plants.

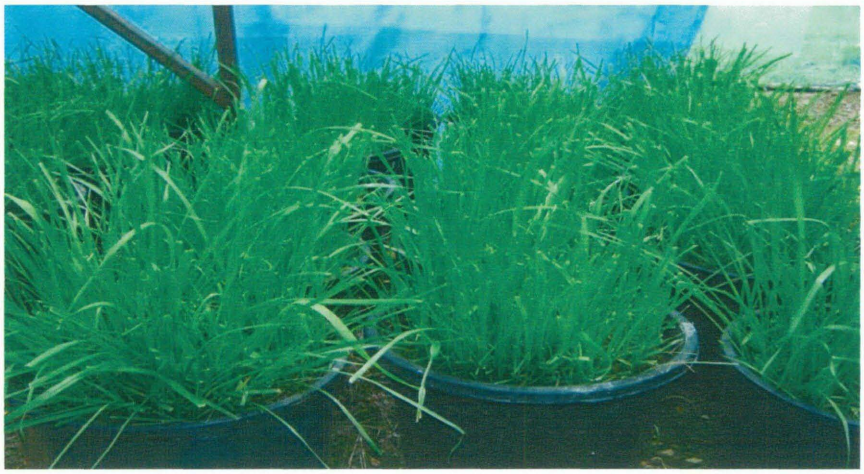


Figure 6-1. Second re-growth of ^{15}N-labelled ryegrass (about 60 d after planting)

6.2.1.2 Experimental animals and diet

Four crossbred sheep fitted with rumen and abomasal cannulae were transferred from the paddock to individual metabolism crates in a temperature controlled, continuously lit room (see Figure 6-2). They were allowed about 2 weeks to become familiar with their new environment and to the experimental diet, i.e. 800 g lucerne hay (DM = 84.7%, N = 3.5%) given each day in equal portions at hourly intervals by means of an overhead, automatic belt feeder slowly feeding. This was used to promote steady state conditions in the rumen. This feeding routine was maintained throughout the experimental period.

Figure 6-2. Fistulated animals in metabolism cage

6.2.1.3 Experimental design and procedure

This experiment was carried out in 2 five-day periods using a 2×2 cross-over design. All sheep received a continuous intraruminal infusion of Cr-EDTA (2.73 mg Cr/ml) throughout each of the 2 periods. Cr-EDTA and acid-insoluble ash (AIA) in abomasal contents were used as fluid phase and solid phase markers, respectively. On Day 1 of each five-day period, 2 sheep were allowed to ingest 60 g ^{15}N-labelled ryegrass in a single meal at about 09:00 h (Treatment A). Chemical composition and ^{15}N enrichment is shown in Table 6-1.

Table 6-1. Fresh ^{15}N-labelled chemical compositions and enrichment (%)

Period	DM	N	^{15}N enrichment	WSC	NSC	Starch
1	10.2	5.1	8.9	2.0	4.7	2.7
2	12.3	4.9	9.7	3.3	5.0	1.7

The other 2 sheep received an intraruminal injection of 40 ml of $^{15}NH_4Cl$ solution (enrichment 99% ^{15}N) which provided 33 mg ^{15}N that was mixed with 50 ml of Co-EDTA (5.92 mg Co/ml) complex and used as liquid phase marker for estimation of rumen volume and fluid outflow rate (Treatment B). Sheep offered ^{15}N fresh ryegrass were given an equivalent intraruminal injection of deionised water. Between periods 1 and 2 there was 2 days as rest period which animals were fed basal diet and had free access to drinking water. The same treatments were applied to the other 2 sheep in Period 2. The allocation of treatments was as shown in Table 6-2.

Table 6-2. Allocation of animals to treatments in Period 1 and Period 2

Sheep / Period	1	2	3	4
1	A	B	A	B
2	B	A	B	A

At the start of experinment, about 08:00, ruminal dosing with $^{15}NH_4Cl$ and Co-EDTA was done by pouring the solution into the rumen via a large plastic funnel connected to a stiff plastic tube. This tube could be pushed into the rumen contents and directed to different parts of the rumen to promote more rapid mixing of the tracers throughout the rumen contents. After all the solutions had been dispensed, the container and funnel were rinsed with about 20 ml water and the tube was withdrawn carefully from the rumen contents to ensure quantitative delivery of all tracers.

After the sheep had received the tracers, samples of rumen fluid (25-35 ml) were obtained at increasing intervals for 2 d, viz. after 30, 75, 165, 300, 450, 600,

840, 1320, 1500, 1900, 2880 min. Before administration of tracers, one sample of rumen contents were taken to enable DM, N, soluble protein, ammonia and NP-NAN (non-protein, non-ammonia N) concentrations and background ^{15}N abundance in total N to be determined.

Rumen fluid was withdrawn into 60-ml syringes connected to a tube that passed through a rubber bung at the mouth of the cannula into the ventral sac of the rumen where it was connected to a specially constructed stainless steel cage covered with a polyester monofilament fabric (PeCAP, 100-m mesh, Tetko Inc., Elmford, NY) to filter large particles from the fluid during collection. The sampling probe was washed thoroughly between samples by injecting water after each sampling, and by withdrawing about 40 ml of rumen fluid and returning it via the sampler before each new sample was collected. The pH was determined instantly on a sub-sample of 5 ml rumen fluid immediately after collection. The remainder was placed in a centrifuge tube held in ice pending transport to the laboratory for high-speed centrifugation (within 1 h). During the first sampling day, one sample of whole rumen contents was collected just before ^{15}N was ingested or administered and three more samples were then taken after ^{15}N administration to enable ^{15}N clearance from the rumen to be determined. Representative sub-samples of the basal diet (lucerne hay) and of the ^{15}N-labelled fresh forage samples were retained for analysis so that the quantity of total N and ^{15}N administered or ingested could be accurately determined.

6.2.1.4 Urine collection and analysis

Urine was collected every morning from each sheep into 5 L plastic buckets containing 50 ml of 10% (v/v) H_2SO_4 over 24 h periods for 4 d after the administration of ^{15}N. The bucket contents were transferred to a 2 L measuring cylinder and its volume was recorded. Then the contents of the cylinder were then diluted with cold tap water up to 2 L, and pH was recorded. If necessary a few drops of 10% (v/v) H_2SO_4 were added to the diluted urine to lower the pH to below 3. A 50 ml sub-sample was taken and frozen for subsequent analysis of allantoin by the colorimetric method described in Section 3.2.6.1.

6.2.1.5 Sampling and preparation of digesta

Sampling of the whole rumen content was done according to the procedure mentioned in Section 3.2.2.1. Abomasal digesta were collected via the abomasal cannula at 3, 8, 14 and 24 h after the start of the experiment. When the bung was removed, any digesta trapped in the cannula stem was scraped out and discarded. A John vial was placed underneath the stem and digesta were allowed to flow into it. A smooth plastic tube was used for removing any blockage in the cannulae and gently swirling the abomasal digesta. Usually, 50 g of digesta was collected in 2-4 min. The digesta were stored in the collection container at –18 °C until analysis. The abomasal digesta samples were then allowed to stand at room temperature for about half an hour. A fraction with only small particulate matter was removed from the top of the sample and transferred to another container and the fraction with more concentrated solids was left in the original container (see Section 3.2.3.1).

6.2.1.6 *In situ* experiment

It was decided that the degradation characteristics of the fresh ryegrass ingested by the sheep in this study should also be evaluated by means of a rumen *in situ* experiment. This experiment was done using material cut within the rest period between period 1 and period 2 of the *in vivo* experiment. The *in situ* experiment provided results for the *in situ* disappearance rate of fresh ryegrass DM, N and ^{15}N from nylon bags in the rumen of sheep given 800 g/d chopped lucerne.

The objective was to compare *in situ* disappearance rates of N and ^{15}N of fresh labelled ryegrass with the results for *in vivo* degradation of ingested ryegrass in the rumen of sheep fed dry chopped lucerne hay under nearly similar conditions. In this case, the amount of ^{15}N placed in the rumen in all of the bags was approximately similar to the amount ingested in the *in vivo* experiment. Thus, the rumen microbes in the bags could be expected to be labelled to about the same extent as those in the rumen of the sheep ingesting labelled ryegrass.

Fresh ryegrass was harvested within 3 d of the *in vivo* experiments and evaluated by the *in situ* method in Border Leicester x Merino crossbred wethers lambs (BW= 27.7 ± SD 2.3). The sheep were kept in individual pens in the animal house at the University of New England. They had free access to drinking water and received a basal diet of chopped lucerne hay (800 g) daily at about 0900 h.

About 30 min after harvesting, 10 g of fresh ryegrass samples (DM = 12.3%, N = 4.8% DM, ^{15}N enrichment = 8.3%) were chopped by scissors to approximately 5 mm lengths and weighed into nylon bags (size 150 mm x 80 mm, pore size 44 μ) together with a marble (about 5 g). The marble was added to ensure that the bags remained in the rumen digesta during incubation and to facilitate the removal of the bag from the rumen when the desired incubation periods had been ended. The bags were then tied firmly with a monofilament fishing line before being suspended in the rumen of the 5 animals through the rumen fistula.

Six bags were placed in the rumen of each sheep simultaneously and removed after 0, 3, 7, 12, 21, and 33 h after incubation. Immediately after withdrawal from the rumen, the bags were put into a bucket containing cold water and feed particles were washed off. Then they were transferred to another bucket and cleaned under running tap water to ensure coarse particles trapped around the mouth of the bag were removed. The bags were then washed thoroughly with running cold tap water while being squeezed gently until no more visible colour came from the bags and their contents. Bags designated 'time zero' were never placed in the rumen, but were otherwise treated in exactly the same way as bags placed in the rumen. All washed bags were gently squeezed, placed in an aluminum tray and dried in a forced-draught oven at 65°C for 48 h. After drying, bags were cooled in a desiccator and weighed. and the residues were used for N and ^{15}N analysis. DM loss was determined. Laboratory analysis and calculations were done as described in Sections 5.2.4 and 5.2.5.

6.2.2 Laboratory analysis

6.2.2.1 Ammonia, NAN and bacterial enrichment

The rumen fluid samples were centrifuged (20,000 x ***g***, 15 min, 4°C, Beckman Model J2-21M) after transfer in ice from the animal house to the laboratory (within 1 h of sampling). A sub-sample (10 ml) of the supernatant fraction was acidified with 0.25 ml 18 M H_2SO_4 and stored at -18°C pending analysis for ammonia and NAN and about 8 ml was added to pre-tared disposable tubes containing 0.8 ml 65% (w/v) trichloroacetic acid (TCA) solution to precipitate soluble proteins. The exact mass of fluid added was determined by weighing the tube before and after the addition of fluid. The tubes were well mixed and stored at 4°C for at least 24 h, to enable the soluble proteins to be completely precipitated (see Section 3.2.8.8).

Bacteria in the top layer of the solid fraction after centrifugation were resuspended in 2-4 ml of the supernatant using a Pasteur pipette and transferred to another centrifuge tube (30 ml) that was then filled with deionised water. The refilled tubes were centrifuged again (20,000 x ***g***, 15 min, 4°C). The supernatant was discarded and the top layer which was almost pure fluid-phase bacteria was resuspended in 3-4 ml of water, transferred to small tubes (5 ml), and stored at -18°C. (This procedure was sufficient to dilute the ammonia and other soluble N from the original rumen fluid to negligible concentrations in the bacterial suspension). The frozen samples were later freeze-dried and the dried bacterial sample was pulverized to a fine powder by adding 2 glass beads into the same tubes and agitating them using a Vortex mixer. A sub-sample (around 2 mg) of the dried ground bacterial sample was transferred into a weighed tin capsule. The capsule was then re-weighed to give a sample of known weight for analysis of bacterial-N and ^{15}N enrichment using the Carlo-Erber-IRMS. The enrichments of ammonia-N, peptides and amino acids, soluble protein and bacterial-N were determined as described in Section 3.2.8.4.

6.2.2.2 Rumen liquid volume and liquid outflow rate (rumen kinetics)

The Co concentration in thawed rumen fluid supernatant solutions was determined using an inductively Coupled Plasma Optical Spectrometer (ICP-OES) with wavelength range covering 175-785 nm after the samples were digested with perchloric acid ($HClO_4$)/ H_2O_2. The readings were interpolated from a standard curve generated from samples representing various dilutions of a Co standard (Ajax Finechem, Seven Hills Australia).

In order to check the reliability of this method and therefore find out its reliability, some of the rumen fluid samples were spiked with standard Co solution, and subjected to the same digestion process as the other samples. The rate of dilution of Co in the rumen was well-described by the following equation:

$$C_t = C_0 e^{-kt} \qquad (12)$$

where C_t and C_o are the concentrations of Co at time (t) and zero time, respectively, *e* is the natural logarithm base and k is the rate constant (/min).

Rumen fluid volume and outflow rate were then calculated as follows:

Rumen volume (RV) (L) = Dose injected (mg Co)/C_0

Outflow rate (L/day) = RV × k × 1440

where k is the rate of passage

Digesta phases from abomasal samples were recombined mathematically (Faichney 1975) to estimate the flow rate of true digesta and digesta constituents through the abomasum.

6.2.2.3 Enrichments and curve fittings

Enrichments of all samples were calculated by subtracting the background abundance from the abundance of the corresponding pre-dosing sample. The

enrichments were then normalised (by linear scaling) to represent 1 mmol ^{15}N (above natural abundance) administered (either as ^{15}N-ammonia or ^{15}N-ryegrass) into the rumen. The normalised enrichment data (E_t) versus time for bacterial-N, soluble protein-N, ammonia-N and NAN in rumen fluid supernatant fractions were then fitted by curves representing the sum of two exponential functions using SigmaPlot software v.10 (SigmaPlot 2006).

$$E_t = a * \exp(-b * t) + c * \exp(-d * t) \qquad (13)$$

where a, b c, d represent the coefficients and t is time (min).

The bacterial curves were fitted first with no weighting factor applied when minimizing the residuals but with the constraint that the curve must start from zero enrichment ($E_t = 0$; a=-c). The remaining build-up and decay curves (soluble protein and NAN) were similarly fitted, but with an additional constraint that the terminal rate constant (d) must be the same as that for the fitted bacterial N curve. For each ammonia-N decay curve, the terminal exponent was also constrained to be the same as the terminal slope of the bacterial curve. In addition, the zero-time intercept of (a + b) was constrained to that required to give the ammonia compartment size determined from the rumen volume (obtained by dividing the dose of Co-EDTA by the intercept of curve describing Co-EDTA concentration v. time) and the mean ammonia concentration in rumen fluid over the period from 09.00 h and 15.00 h. The residuals for the ammonia curves were weighted by $1/E_t$.

6.2.2.4 Calculations of N compartment sizes and fluxes

The quantity of N present at time zero was estimated from the water: N ratio in whole rumen contents and the water volume (L) obtained from the dilution of intraruminally administered Co-EDTA.

The rates of total flux and net flux of ^{15}N, and the fractions of bacterial-N derived from rumen ammonia were calculated by the methods given by Nolan and Leng (1974). The fraction of the ^{15}N in the ingested ryegrass that subsequently entered the rumen fluid ammonia compartment was used as an index of ruminally

degradable protein in ryegrass, although it was recognized that NAN degradation intermediates (*e.g.* peptides or amino acids) would also have been likely to be assimilated by microbes. This fraction was determined from the rate of flow of ^{15}N through the ammonia compartment after administration of ^{15}N-labelled ryegrass. The latter was taken to be the product of the area under the ammonia curve and the rate of flux of ammonia through the ammonia compartment determined when the same animals received ^{15}N-ammonia.

The DM, N and ^{15}N enrichments of the 3 samples of whole rumen digesta taken after the sheep ingested the ^{15}N-labelled ryegrass were fitted by a single-exponential curve similar to that used for describing Co-EDTA dilution with time. Measurement of and total N, ^{15}N and DM disappearance from the rumen were determined during the first sampling day. Estimates of total DM and N pool sizes in whole rumen contents were made by using the rumen fluid volume (kg) determined from Co-EDTA dilution, and the mean DM (g/kg water) and N (g/kg DM) concentrations in the samples of whole rumen contents.

6.2.2.5 *In situ measurement*

Results were analyzed as described in Section 5.2.4 and 5.2.5.

6.2.2.6 Statistical model and methods

Data were analyzed using the general linear models (GLM) procedure of (SAS 2003) with the following statistical model:

$$Y_{ijk} = \mu + Treat_i + Sheep_{ij} + Period_{ijk} + e_{ijkh}$$

where Y_{ijk} = the performance during the k^{th} period of the i^{th} treat and j^{th} sheep, ($i = 1,2$; $j = 1, 2, .., ni$; $k = 1,2$), μ = the overall mean effect, $Treat_i$ = the effect of the i^{th} treat ($i = 1,2$), $Sheep_{ij}$ = the effect of the j^{th} sheep on the i^{th} treat, ($j = 1,\ldots 4$), $period_{ijk}$ = the effect of the k_{th} period ($k = 1,2$), e_{ijk} = the random error.

6.3 Results

Dry matter intakes of the 4 sheep were 600 g/d. Based on an analysis of whole rumen contents sampled on 3 occasions during the tracer experiments, the mean DM pool size in mixed rumen contents (each based on the analysis of 8 samples) was 709 g (SD 32.3) and the mean total N pool size was 26.6 (SD 2.34) g. The flow of DM to the abomasum was 421 g/d (SD 36.7).

The curves for Co concentration (mg/l) *v.* time (h) in the rumen fluid of two sheep which received ^{15}N-ryegrass or $^{15}NH_4Cl$ following the intra-ruminal injection of Co-EDTA are presented in Figure 6-3.

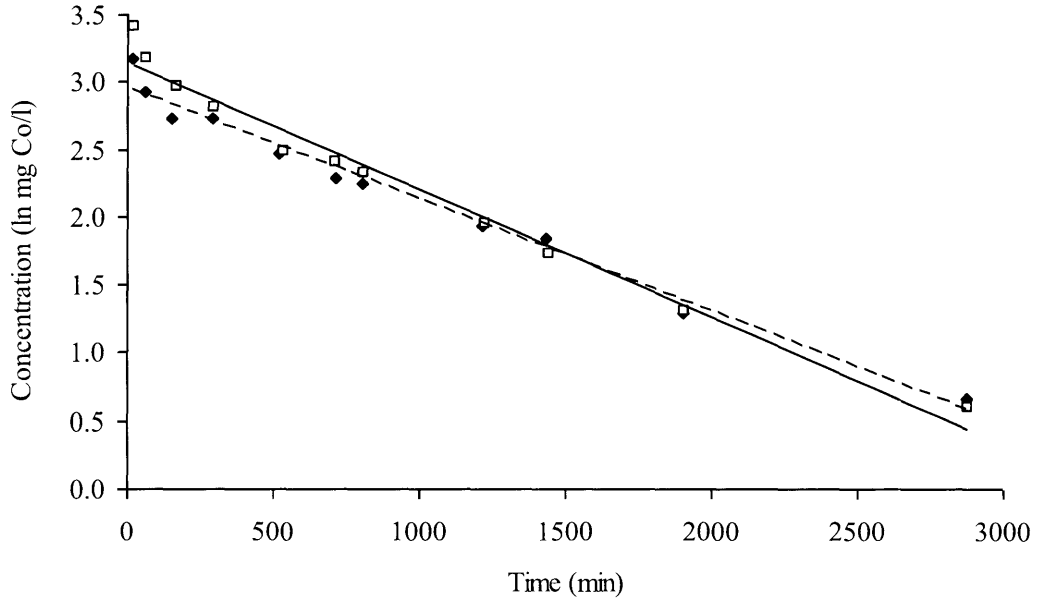


Figure 6-3. The rumen concentration of Co (ln mg/l) with time (min) following an intra-ruminal injection of Co-EDTA in Sheep 1 and Sheep 4 receiving ^{15}N ryegrass (—) and $^{15}NH_4Cl$ (---) in Period 1, respectively.

The mean rumen water volume was 5 L and rates of rumen water outflow, abomasal fluid flow and net water addition between the rumen and abomasum were 5.68, 6.50 and 0.83 L/d, respectively.

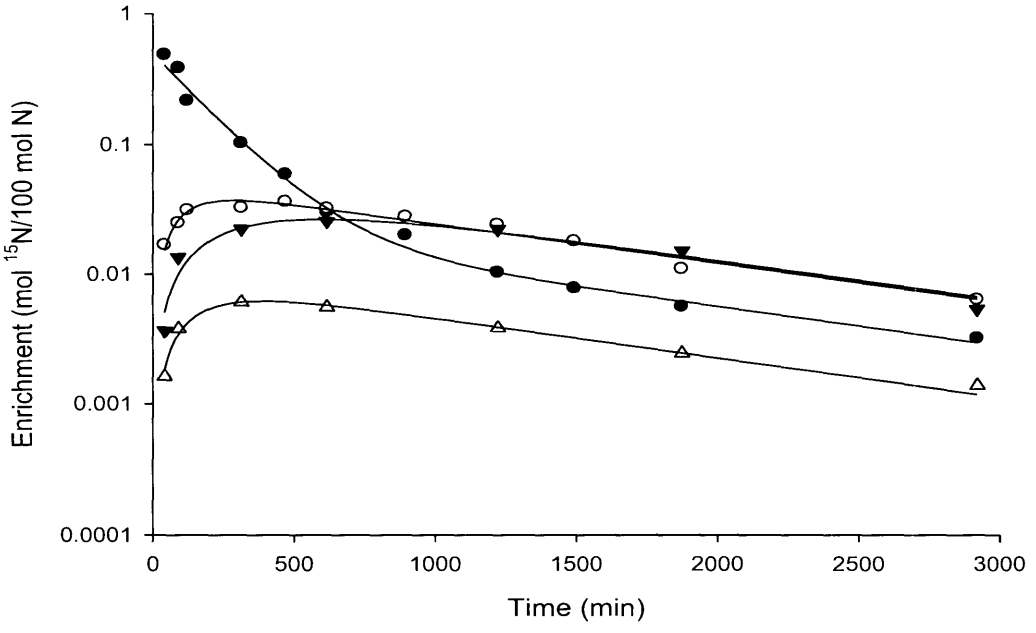
6.3.1.1 N intake and flows through the gut

The dietary N intake from the chopped lucerne hay was 20.7 g N/d. An additional 0.31 and 0.30 g N was ingested as fresh ^{15}N-ryegrass in the first and second periods, respectively. The corresponding value for animals on $^{15}NH_4Cl$ was 0.33 g N on the experimental days in both periods.

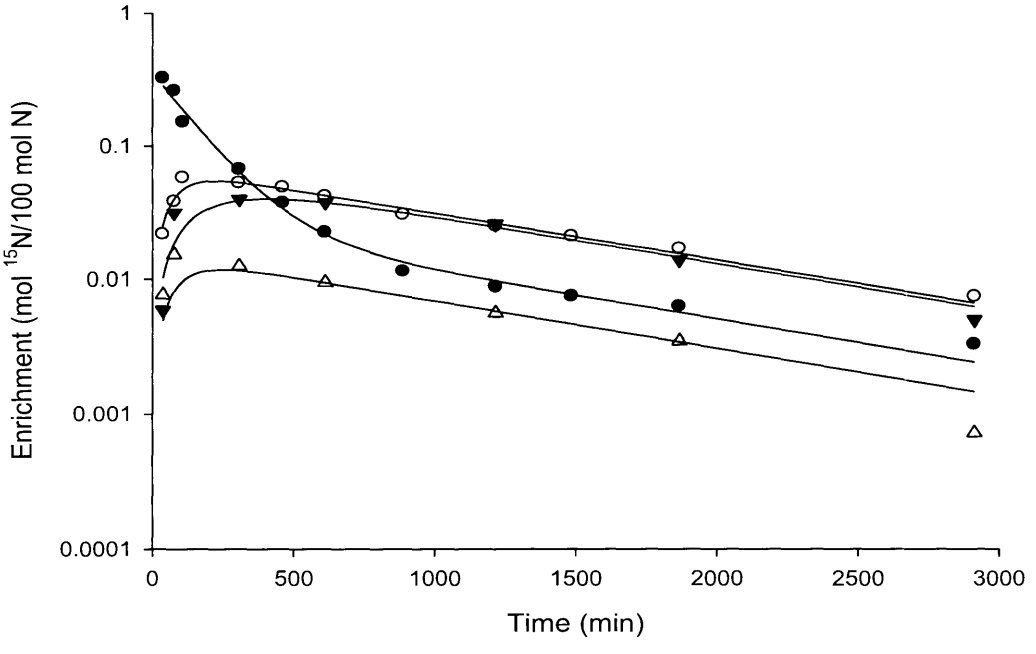
In the present study total N flowing to the abomasum was not determined and so values given by Nolan (1975) for sheep ingesting the same quantity of lucerne chaff (800 g/d) were used. The flow of microbial N in abomasal digesta was determined from the rate of excretion of purine derivatives in the urine (10.9 g N/d, see Table 6-3) and was similar to the value (11 g N/d) given by Nolan (1975). Ammonia absorption, calculated assuming that ^{15}N not present in digesta flowing from the rumen was lost by absorption of ^{15}N-ammonia (4.3 g N/d), was similar to that (3.5 g N/d) given by Nolan (1975).

The ammonia concentrations in rumen fluid were not affected by the source of ^{15}N tracer and mean values was 16.3 mol/l (SD1.1) or 228 mg N/l (15 SD). The rumen volume estimated by Co-EDTA dilution was 5 L. Therefore, the rumen ammonia compartment size was 1.1 g N.

The actual (*i.e.* unscaled) amount of ^{15}N ingested by sheep as fresh ryegrass was 2.1 mmol, and the actual (*i.e.* unscaled) amounts of $^{15}NH_4Cl$ administered was 2.18 mmol. However, the results presented below and the discussion are based on values for enrichment scaled to 1 mmol ^{15}N ingested in fresh ryegrass or administered intraruminally as $^{15}NH_4Cl$. About 5% of the total N in the fresh ^{15}N-ryegrass that was administered to the sheep was present in the form of ammonia. Two randomly selected sets of enrichment *v.* time results for the period after ^{15}N administration and the two-exponential curves fitted to these results are given in Figure 6-4.



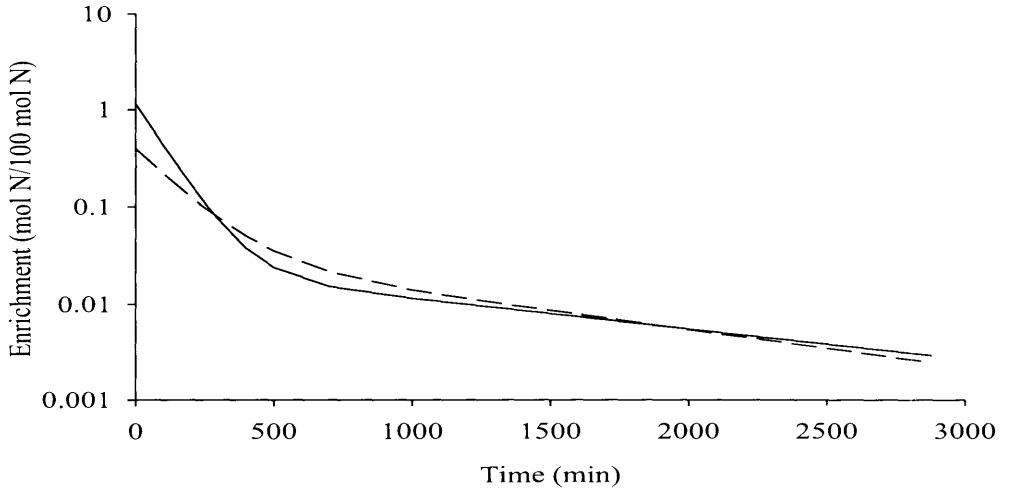
(a)



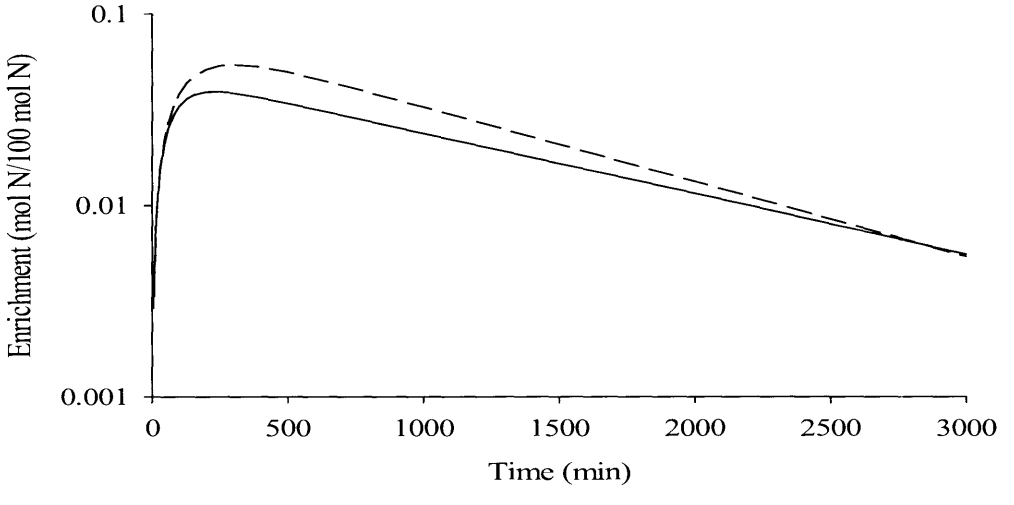
(b)

Figure 6-4. Enrichments (y axis; mol ^{15}N/100 mol total-N) in rumen fluid ammonia (•), rumen fluid bacterial-N(○), peptides and amino acids (△) and soluble

proteins (▼) versus time (x axis; min) after intra-ruminal administration of 1 mmol ^{15}N as ^{15}N-labelled ammonia (a) or ^{15}N-labelled ryegrass (b). The lines represent fitted curves with two exponential functions. In addition to the curves shown in Figure 6-4, there are 4 other similar sets of fitted curves. The results for all 4 sheep in the period after they ingested 1 mmol ^{15}N in ryegrass or were dosed intra-ruminally with 1 mmol $^{15}NH_4Cl$ were fitted, and then the exponential coefficients were averaged to provide the equations for 4 composite curves for each pool sampled (Figure 6-5).

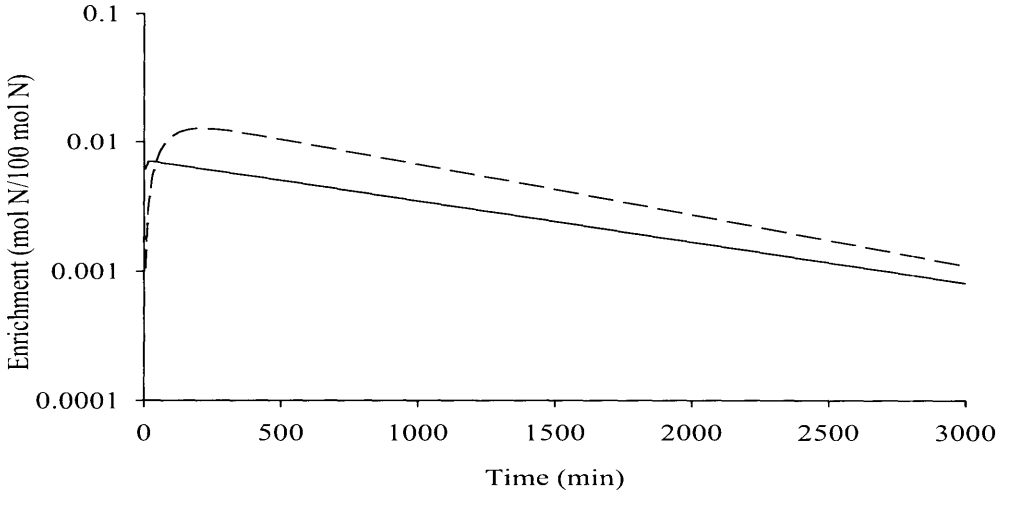


(a)

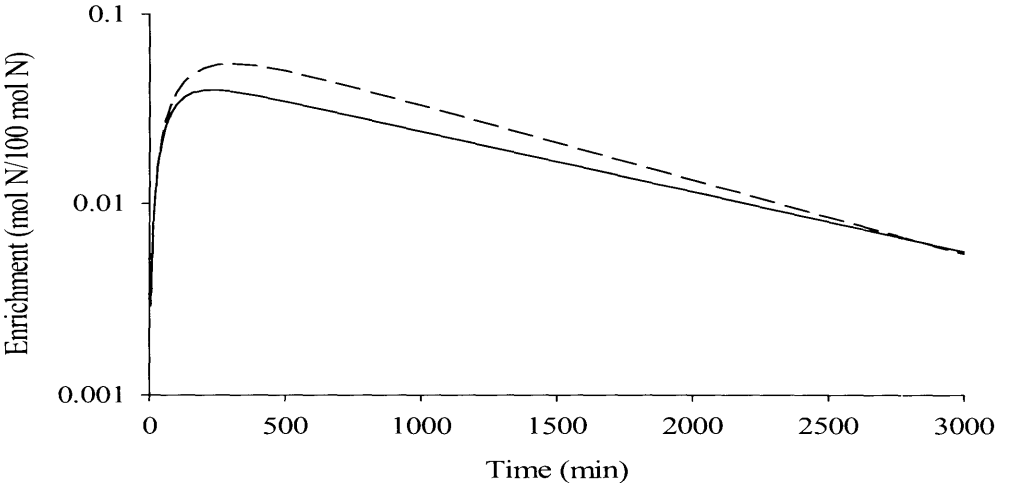


(b)

Figure 6-5. Composite curves of enrichment of ammonia (a), bacteria (b) in the rumen of animals received fresh ^{15}N labelled ryegrass (----) or $^{15}NH_4Cl$ (—)



(a)



(b)

Figure 6-6. Composite curves of enrichment of peptides and amino acids (a) and soluble proteins (b) in the rumen of animals received fresh ^{15}N labelled ryegrass (----) or $^{15}NH_4Cl$ (—)

Figure 6.4 shows that after sheep ingested 1 mmol ^{15}N–labelled ryegrass, their rumen ammonia compartment became quickly and appreciably labelled with ^{15}N, although the enrichment over the first 4-5 h was lower than occurred when

1 mmol ^{15}N-labelled ammonia was administered, i.e. about half of the ryegrass N quickly appeared as ammonia. Bacterial N (see Figure 6-5) was also rapidly labelled after labelled ryegrass or ammonia entered the rumen. Peptides and amino acids in rumen fluid were also rapidly labelled (see Figure 6-6) with the label appearing almost immediately from ammonia; the extent of labelling in rumen fluid peptides and AA after ingestion of fresh ryegrass was, however, greater than from ammonia. Soluble protein in rumen fluid was also labelled to a greater extent when ryegrass was processed in the rumen.

The parameters for the composite curves generated by dosing the sheep with $^{15}NH_4Cl$ were also used to calculate the size of the ammonia pool and the N fluxes. Similar calculations were not able to be made for the sheep ingesting ^{15}N-labelled ryegrass because there was no readily sampled primary compartment. The kinetics of N in the rumen of sheep received $^{15}NH_4Cl$ is shown in Table 6-3.

Table 6-3. kinetics of N in the rumen of sheep that received $^{15}NH_4Cl$

Item	$^{15}NH_4Cl$
Mean ammonia concentration (mg N/l)	228± 20
N Intake (g/d)	
From chopped lucerne hay	20.7
From labelled ryegrass or ammonia	0.32
NH_3-N pool, g N	1.15
Total flux, g N/d	13.47
Irreversible loss, g N/d	10.74
Recycled NH_3-N g N/d	2.73
Enrichment from NH_3-N pool, %	
Bacterial N	0.34
Peptides and amino acids N	0.29
Soluble proteins N	0.05
Microbial flow to duodenum (g N/d)	10.9

In the rumen of the sheep dosed with $^{15}NH_4Cl$, the total ammonia flux through RF was 0.7 × N intake and 1.3 × net flux and about 20% of the ammonia-N leaving the RF ammonia compartment was then returned to it. It is supposed that

nearly all of the recycling occurred in the rumen, e.g. by bacterial assimilation of ammonia followed by protozoal predation or lysis for other reasons and ammonia release from protozoa or microbial cells lysis. Although similar values were not able to be determined when the sheep ingested labelled ryegrass, the values are likely to be similar.

Based on the ratio of the area under the rumen bacterial-N curve to that under the rumen fluid ammonia curve for the sheep receiving $^{15}NH_4Cl$, 34% of the N in fluid-phase bacteria was derived from ammonia, and the remaining 66% was therefore derived from NAN. Based on allantoin excretion in urine, the net flow of N through the bacterial pool was about 10.9 g N/d, so bacteria obtained 3.7 g N/d from ammonia and about 7.2 g N/d from peptides and/or amino acids. The corresponding bacterial enrichment curve for the sheep that ingested ^{15}N-labelled ryegrass was considerably higher than that for sheep given the same dose of $^{15}NH_4Cl$, confirming that the rumen bacteria obtained ^{15}N from sources other than ^{15}N-ammonia for their protein synthesis, i.e. from ^{15}N-labelled NAN intermediates such as peptides and amino acids released from the labelled ryegrass.

To further determine the sources of N used by bacteria, a curve was derived by combining results from the 4 sheep that represented the passage of ^{15}N from the ingested ^{15}N-labelled ryegrass into the bacterial pool via two separate routes, *viz.* ammonia and non-protein, NAN. Then, by subtracting the ^{15}N assimilated into bacteria solely as ammonia (scaled results obtained when the sheep received only ^{15}N–ammonia), a second curve was derived to represent the ^{15}N flow into the bacterial pool from non-ammonia sources, i.e. NP-NAN. Finally, the flow of ^{15}N through the fluid phase NP-NAN pool (measured rumen volume in L multiplied by measured NP-NAN concentration in mg N/L) was calculated to check whether this rate of flow of ^{15}N through the rumen fluid pool of NP-NAN occurred. The 3 curves are shown in Figure 6-7 .

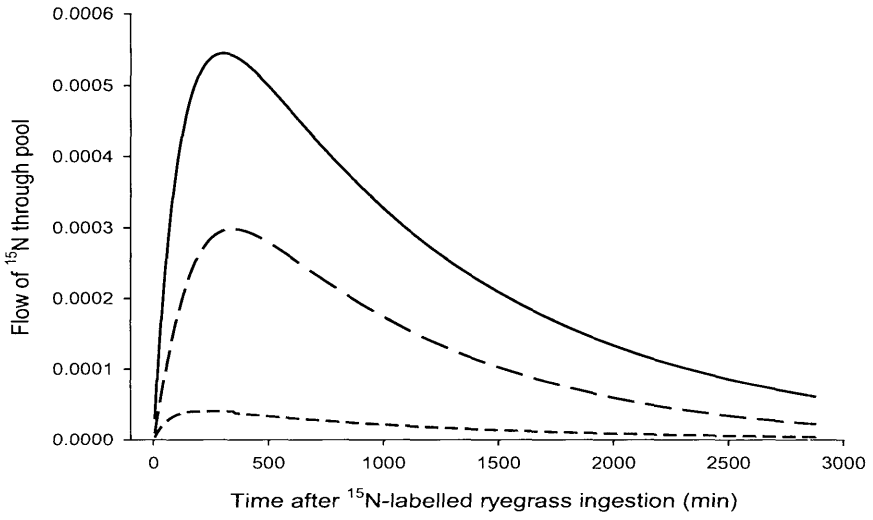


Figure 6-7. Flows to the abomasum of ^{15}N in the rumen bacterial pool that arose from both peptides/amino acids and ammonia after sheep ingested 1 mmol ^{15}N-labelled ryegrass (—), ^{15}N entering bacteria only from non-ammonia N sources (- -), calculated by deconvolution to remove any flow of ^{15}N from ryegrass via ammonia to bacteria) and the actual flow of ^{15}N through the peptide/AA pool in mixed rumen fluid, obtained by direct measurement of pool size and enrichment over time (--).

It is clear from a comparison of the top and middle curves that a major component of bacterial N arises from NP-NAN as well as from ammonia. Comparison of the middle and lower curves indicates that far more ^{15}N-labelled NAN was assimilated by rumen bacteria than passed through the mixed fluid-phase pool of peptides/amino acids in the rumen. This indicates there was a direct route of uptake of non-protein NAN from ryegrass into rumen bacteria that did not pass through the mixed rumen fluid, i.e. there was direct transfer of ^{15}N from ryegrass matter to bacteria suggesting that bacteria assimilated plant N at their sites of attachment.

For the sheep that were dosed with $^{15}NH_4Cl$, the curves in Figure 6.4 show that almost 100% of the ^{15}N administered passed out of the ammonia compartment over the next 2 d. About 62% of the ^{15}N administered to the sheep that ingested ^{15}N-labelled ryegrass passed through the rumen ammonia compartment in the same

period, indicating that there was extensive degradation and release of ryegrass ^{15}N into the rumen contents. Notably, some of the ^{15}N from ryegrass was rapidly degraded to rumen ammonia within 2-3 h of ryegrass ingestion (only 5% of the total N in the ryegrass ingested was initially present as ammonia).

To obtain a more complete understanding of the extent of degradation of the crude protein fraction of fresh ryegrass in the rumen, the flows of ^{15}N via all routes out of the rumen were determined. For example, the flow of in ^{15}N out of the rumen in bacteria was calculated by multiplying the flow rate (g N per min) via the enrichment of the N at each minute of the day and summing the values for 1440 minutes to represent the amount flowing for the day. Similar calculations were made for ammonia absorption across the rumen wall and for the outflows in peptides and amino acids and ammonia in fluid outflow to the abomasum. At the end of the second day after the sheep ingested labelled ryegrass, 105% of the ^{15}N ingested was accounted for. A value > 100% is possible because the calculation takes no account of ^{15}N that may have been added to the rumen during the day by recycling from endogenous sources.

6.3.1.2 N turnover of whole rumen contents

The enrichments of total N in the rumen were recorded on 3 occasions after the sheep ingested ^{15}N-labelled ryegrass or were dosed with $^{15}NH_4Cl$, enabling the fraction of the dose remaining in the rumen to be calculated (see Figure 6-8).

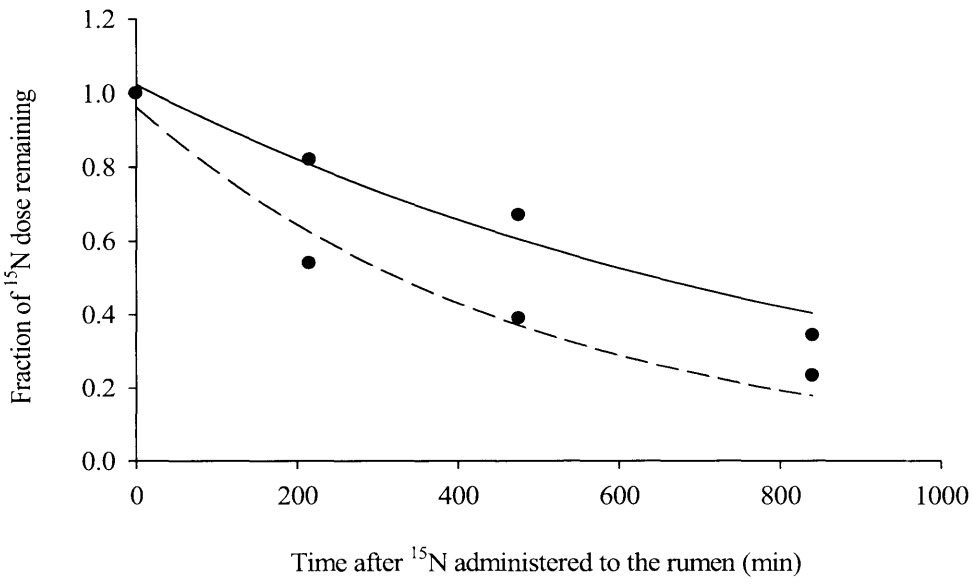


Figure 6-8. Fraction of ^{15}N remaining in the total N of mixed rumen contents after sheep ingested 1 mmol ^{15}N as ^{15}N-labelled ryegrass (---) or received an intraruminal dose of ^{15}N as $^{15}NH_4Cl$ (—). The results were fitted by exponential decay curves with two parameters

The rate constants for the fitted curves were 1.53 per d and 3.03 per d, respectively, when ryegrass or $^{15}NH_4Cl$ were the sources of ^{15}N. If it is assumed that the N sampled represented the total N pool in the rumen, the corresponding N flux rates (pool size x rate constant) through the total N pool in the rumen would have been 40.7 and 80.6 g N/d, respectively. These values are considerably higher than the rate of ingestion of total N. Projection of these curves suggests that, of the 1 mmol ^{15}N initially present in the rumen contents after ingestion of ^{15}N-labelled ryegrass by the sheep or after intraruminal administration of $^{15}NH_4Cl$, about 3.2% and 0.6% would have still been present after 2 d, respectively. The residual plant ^{15}N would have been lower than these values indicate because some recycled ^{15}N would have been present at 24 h.

6.3.1.3 *In situ* results

There were no statistically significant differences between the 3 sheep (as replicates) in the values for DM, N and ^{15}N disappearance during the *in situ* incubation of the fresh ryegrass, so the respective mean degradabilities of fresh ryegrass are presented in Figure 6-9.

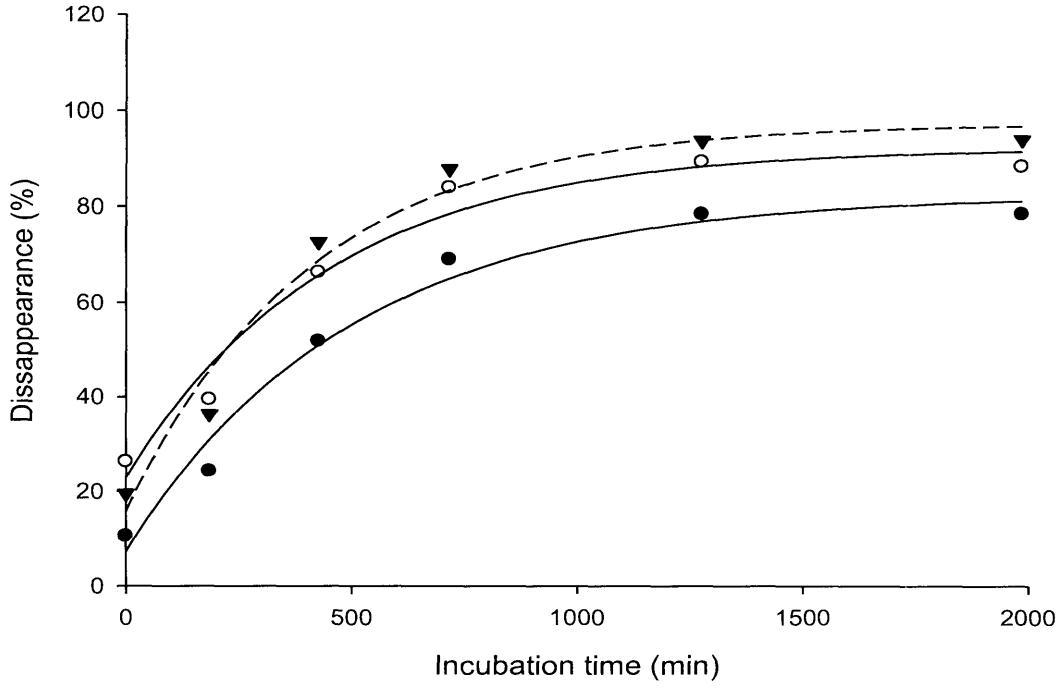


Figure 6-9. *In situ* disappearance (%) of DM (●), N (○) and ^{15}N (▼) for sheep

Results shown in Figure 6-9 showed the same trend as reported in the results of experiment reported in Chapter 5. The ^{15}N removal from the bag represents the loss of ^{15}N ryegrass, but there would still be some ^{15}N present in rumen bacteria. True values for ^{15}N ryegrass N removal will be slightly higher than the ^{15}N values shown here, i.e. close to 100 %. However, *in vivo*, some soluble protein and peptides and AA will leave the rumen before being degraded.

In Figure 6-10 shows both apparent and corrected proportional N disappearance when the fresh ryegrass was incubated *in situ* in sheep, and Figure 6-11 shows the predicted ED of forage N (crude protein) before and after correction for microbial contamination, for various periods of digestion in the rumen.

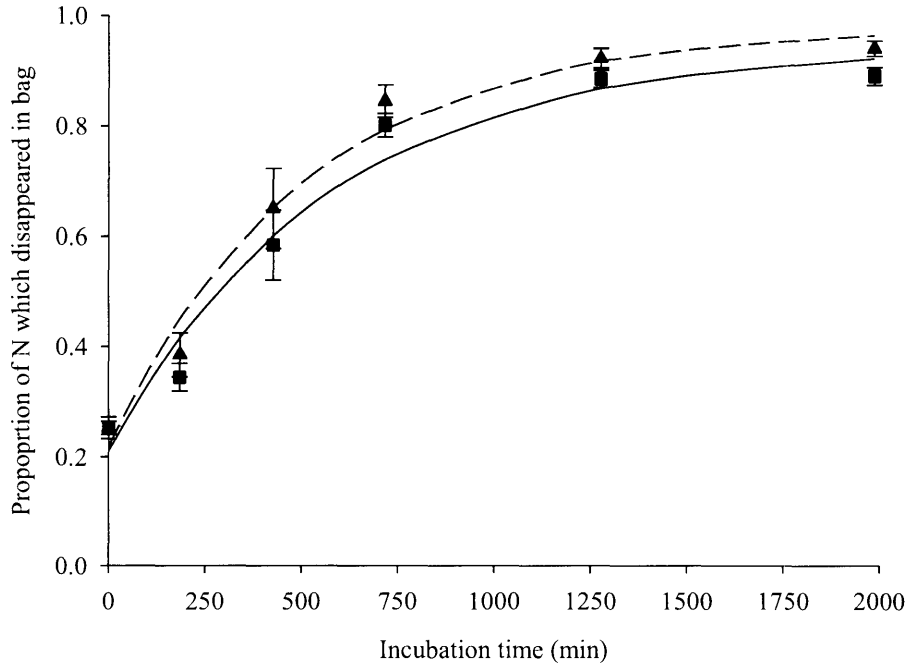


Figure 6-10. Mean (± SE) fresh ryegrass N disappearance (apparent ■, ___ fitted; corrected ▲, ---- fitted) from nylon bags in sheep

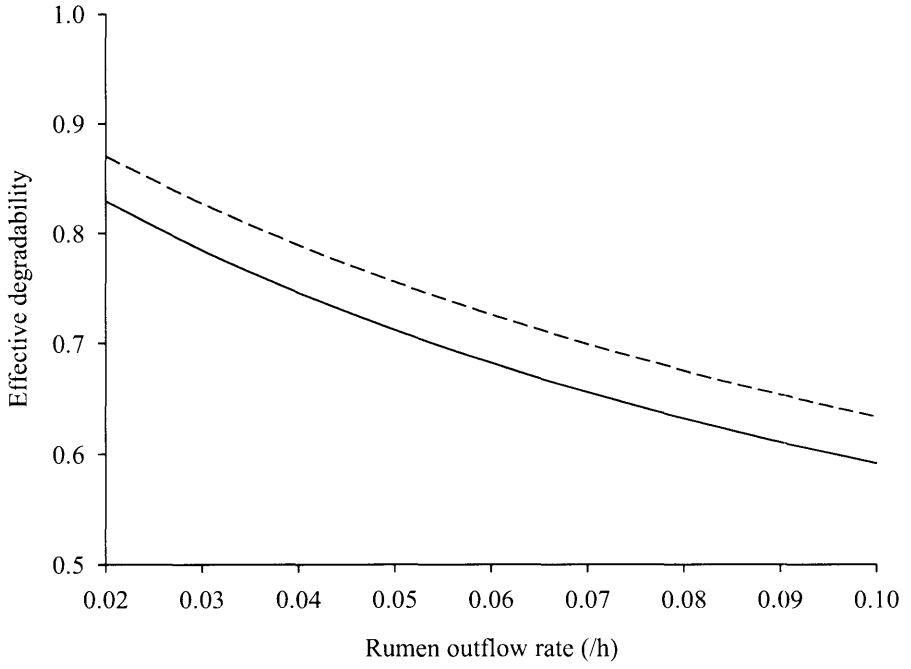


Figure 6-11. ED estimates at increasing ruminal outflow rates

As it shown in Figure 6-10 and Figure 6-11, fresh ryegrass N disappearance and ED of protein in fresh forages underestimated.

6.4 Discussion

6.4.1 Concentration of rumen fluid metabolites

The miminum concentration of ammonia-N in the rumen fluid throughout the experiment was 124 mg N/L which is above the minimum concentration of 50 mg N/L (Satter and Slyter 1974) considered adequate to support microbial growth. Hence, the availability of ammonia-N in the rumen should not have been a limiting factor for microbial growth in the sheep that were given the basal daily ration of 800 g lucerne chaff. The conditions should therefore have been ideal for degradation of the ingested fresh ryegrass. The mean NAN concentration in the rumen fluid from which the bacterial and soluble protein N had also been removed (non-protein, non-microbial NAN) was found to be at least 100 mg/l. This fraction probably contained

peptides and amino acids in rumen fluid. There are relatively few reported estimates of concentrations of soluble protein and peptides and amino acids in rumen fluid, especially for ruminant grazing fresh rather than frozen and thawed forage where the latter could release cell contents and give false values. However, Demeyer and Van Nevel (1980) reported that the concentration of peptide and amino acid in fresh fluid was less than 20 mg N/l, while that of soluble protein was about 40 mg N/l. Their values were probably obtained when sheep were fed once per day, so that their rumen NAN concentration may have been lower than in this study due to rapid deamination of amino acids rather than a higher rate of assimilation for microbial protein synthesis (Wallace and Cotta 1988). It therefore seems likely that the relatively high rumen NAN concentrations observed in this study (100 mg N/L) were most likely because animals were offered feed every hour so that there would have been a more constant release of NAN intermediates from dietary protein.

6.4.2 Flows of DM and nutrients through the abomasum

In this experiment, the mean of DM intake and DM flow through the abomasum was 600 and 421 g/d, respectively. Thus, about 29.8% of the ingested DM was apparently digested in the rumen. However, the true digestibility of DM in the rumen (allowing for microbial DM leaving the rumen of about 140 DM/d) was calculated to be about 53%, which is lower than values obtained in other published studies (SCA 1990). The rumen fluid volume averaged 5 L and water outflow rate calculated from Co-EDTA dilution was 5.7 L/d, equivalent to 4.8%/h and similar to the value often used to determine effective degradability for rumen *in situ* experiments There was a slightly higher flow rate of water (6.5 L/d) through the abomasum suggesting a net post-ruminal water addition, probably as gastric secretions, of about 0.7 L/d: the actual water input may have been higher than the net estimate indicates, if water is also absorbed in this part of the gut of the sheep (*e.g.* in the omasum) as has often been suggested (Holtenius and Bjornhag 1989).

6.4.3 Rumen fluid ammonia flux and recycling

In this experiment, when ^{15}N-ammonia was given as a single injection into the rumen of sheep given a basal diet of 800 g/d lucerne, about 80% of the daily total ammonia flux left the compartment irreversibly, and about 20% was recycled. This recycling was within the range of 10-50% suggested by Nolan (1975) and Damry *et al.* (2001). Higher values (51-57% of the total ammonia flux) have also been reported for defaunated sheep (Koenig *et al.* 2000; Nolan and Stachiw 1979). Intraruminal NH_3 recycling is a measure of the ruminal ammonia-N incorporated into microbial protein or other nitrogenous compounds that later release N into the ammonia-N pool The rumen fluid pool of peptides and amino acids of animals receiving fresh ^{15}N ryegrass also became labelled with ^{15}N, but the rate of appearance of ^{15}N and shape of the curves were different from those for animals receiving $^{15}NH_4Cl$.

In the rumen of animals that received $^{15}NH_4Cl$ in the present study, the percentage of bacterial-N derived from ammonia-N, calculated from the ratio of the area under the bacterial-N enrichment curve to that under the rumen fluid ammonia-N enrichment curve, was about 34%. This indicates that 34% of the N in bacteria was derived from rumen ammonia and the other 66% was derived from NAN, probably in the form of amino acids and peptides. These results are consistent with the values reported by (Nolan 1975). The proportion of bacterial N arising from NAN was higher than suggested by results from other studies in which bacteria derived 50 to 80% of their N requirements from the ruminal ammonia-N pool and 20-50% from NAN (Koenig *et al.* 2000). It should be noted that the enrichment of the bacteria sampled may not have been representative of all bacterial N in the rumen and certainly would not have been likely to be representative of the other micro-organisms, i.e. protozoa and fungi. It is also not known whether soluble protein from rumen fluid was adsorbed on the bacterial surfaces. These results indicate that ryegrass N contributed proportionally more ^{15}N directly to the soluble proteins, peptides and amino acids and to bacterial N than did ^{15}N-labelled rumen ammonia. Comparison of these and other studies suggests that when peptides and amino acids are available, they can be used extensively by rumen microbial populations, but

rumen microbial populations can adapt by using more ammonia when less peptides and amino acids are available.

In the present study, ammonia enrichments indicated that within 30-45 min after the sheep ingested the ^{15}N-labelled ryegrass, there was a sudden and substantial release of ^{15}N-ammonia into the rumen contents (see Figure 6-5) that was not wholly explained by the ammonia present in the ingested plant material (determined to represent 5% of the total N). The rapidly formed ammonia N might have been formed from proteolysis, deamination, amides, amines or other nitrogenous materials that were rapidly converted to ammonia after ingested material entered the rumen. Analyses to determine the concentrations of these nitrogenous materials were not done on the ryegrass samples in this experiment.

The percentage of ^{15}N in ryegrass that passed through the ammonia compartment in the rumen was estimated by assuming that the rates of ammonia entering and leaving the ammonia compartment in the rumen would be similar to that estimated from the results obtained when $^{15}NH_4Cl$ was administered. This percentage (68%) is a minimum estimate of the rumen degradability of ryegrass protein. However, a better estimate of degradability of fresh ryegrass could be obtained by accounting for all ^{15}N leaving the rumen after ^{15}N-labelled ryegrass was ingested. Over the 48 h sampling period, outflows of ^{15}N via ammonia absorption, ammonia in fluid outflow, bacterial N outflow, and outflow in peptides and amino acids, were 22.4, 0.08, 0.71 and 0.04%, respectively. Recovery of ^{15}N in this experiment was therefore estimated to be 105% (SD 3). Recycling of ^{15}N in endogenous secretions provides an explanation for a recovery of greater than 100%.

A third estimate of the degradation of plant material was obtained by monitoring the residual ^{15}N in whole ruminal contents after the animals ingested labeled plant material. Other researchers have sampled rumen contents by emptying the rumen and analyzing the mixed contents but the rumen cannulas in the sheep in this study were too small for this purpose. Obtaining truly representative samples of rumen digesta has been a problem for a long time (Hungate 1966). In this experiment, the sampler used had to be small enough (internal diameter 25 mm) to be inserted through the rumen cannula. Several sub-samples were removed from

different parts of the rumen at each sampling time. Despite these possible limitations, the mean values (n=8) for DM present in the rumen (709 g SD 32.3) and total N (26.6 SD 2.34) were similar to published values (Rodriguez *et al.* 2003). The results obtained by monitoring ^{15}N in whole rumen contents after the sheep ingested labelled ryegrass indicated that degradation was around 77 % at 12 h (cf. 50 *in vitro* and 60% *in situ* methods) and again was close to 100% after 24 h (the curves for net loss of ^{15}N showed that less than 3% of the ingested ^{15}N remained in the rumen contents after 24 h.

Proteases are mainly associated with the cell surface of bacteria (Kopecny and Wallace 1982) and the hydrolysis of proteins to peptides usually occurs extracellularly. The micro-organisms in the rumen may either transport smaller peptides directly into their cells or hydrolyze them further to amino acids extracellularly. As the ruminal bacteria can assimilate both peptides (Chen *et al.* 1987) and free amino acids (Russell *et al.* 1988), protein can be utilized through either peptides or amino acids (see Section 2.8.1). The results of this experiment provide evidence of direct uptake of NAN from degraded ryegrass in forms that are more complex than ammonia. This finding is consistent with the results reported by Hristov *et al.* (2001) who also concluded that peptides and amino acids are extensively used by rumen microbial populations when they are available.

Rumen bacteria assimilate ^{15}N-ammonia in rumen contents for their protein synthesis and become enriched. Release of this enriched bacterial N to the rumen environment would generate enriched NAN samples. However, when the ^{15}N-labelled ryegrass was fed to the animals, its soluble proteins and protein degradation products (peptides and amino acids) would contribute directly to the pool of labelled NAN in the rumen fluid. In this case there is a second source of ^{15}N contribution from products derived from viable or lyzed bacteria labelled by assimilating the labelled peptides, amino acids or ammonia.

Results obtained when $^{15}NH_4Cl$ was administered (Figure 6.5) indicate that 20% of the protein N was derived from ammonia, presumably by rumen micro-organisms, and secreted into the rumen fluid, probably partially as microbial enzymes. It follows that most of the N synthesized into soluble protein (80%) was

from dietary or endogenous sources. This conclusion was supported by the higher overall tracer concentrations that occurred after the same amount of ^{15}N-labelled ryegrass N was ingested by the sheep (Figure 6.5).

6.4.4 Conclusions

There are relatively few studies that provide quantitative information on how proteins from fresh forages are released and hydrolysed *in vivo* after ingestion by ruminant animals. Even when fresh rather than dried materials are used, *in vivo* degradability estimates have often been made using frozen-thawed materials rather than freshly cut material. So these studies were intended to add to information on the degradation of fresh plant materials. The *in vitro* studies described in Chapter 4 showed that freezing can at least in in some situations alter the degradability characteristics of forages. It is therefore recommended that, if *in vitro* or *in situ* methods are used to evaluate the degradation characteristics of forages, fresh samples should be used.

The degradability characteristics of the ryegrass used in this experiment determined *in vivo* and by the rumen *in situ* method and the later results (this Chapter) were similar to those for the fresh ryegrass reported in Chapter 5, i.e. the fresh plant material used in both these studies was highly degradable. All three methods used (*in vitro*, *in situ* and *in vivo*) indicated that fresh forage samples were highly degradable. In this study, about 97% of fresh ryegrass N was degraded in the rumen within one day after ingestion of ^{15}N-labelled ryegrass by sheep.

A further important finding was that the flow of ^{15}N through the rumen fluid pool of peptides and amino acids was much less than the estimate of uptake of these sources of N by bacteria. This finding indicated that not all peptides and AA assimilated by bacteria were derived from peptides and AA pools *in rumen fluid* and supported the idea that proteins from fresh forage are extensively adsorbed onto the surface of rumen bacteria before their breakdown products are utilized.

Chapter 7 General Discussion

7.1 Rumen degradability of fresh forages

A survey of the relevant literature (Chapter 2) revealed that there was little information available on how grazed fresh forages are degraded and utilized in the rumen. The overall objective of the work reported in this thesis was to learn whether there are any differences between different forage species and obtain useful information about the degradabilities characteristics of ingested fresh forages (lucerne and ryegrass) in the rumen. Also, a secondary objective was to determine whether degradation of fresh forages can be accurately estimated on frozen-thawed samples using simpler *in vitro* or rumen *in situ* methods rather than more time consuming *in vivo* studies.

In vitro techniques are relatively simple and readily performed. Estimates of feed protein degradability in the rumen can be based on ammonia production, but may under-estimate N degradability because micro-organisms use some of the ammonia produced for cell growth and also assimilate non-protein NAN intermediates. However, a correction was made by predicting microbial growth and microbial assimilation in the incubation flasks using VFA production and stoichiometry principles. The degradability of feed N was increased as a result of energy addition, suggesting that additional energy enhanced proteolytic activity, probably by increasing numbers of microbes or increasing the proteolytic activity of the microbial population, or both. Results showed that placing samples in an ice bath after sampling was not adequate to stop fermentation quickly. In future, other

methods such as placing the sample in liquid nitrogen or adding chemicals like sodium azide could be used for this purpose. However, it is necessary that any chemical used should stop chemical reactions without affecting the integrity of the sample.

In this experiment it was found that potential degradabilities of OM and CP were different between lucerne and ryegrass, and in the early hours of the incubation, lucerne was degraded more rapidly than ryegrass. When different preparation methods were applied to the forage samples, the fermentation patterns of frozen-thawed samples were different to the fresh samples and addition of starch boosted the amount of fermentable substrate present in the incubation media. At the end of the 12 h incubation, about 50% of the OM was fermented, and fermentation was apparently approaching an asymptotic value. There could be a number of reasons why fermentative activity decreased after 12 h. One reason might be that, after dilution of inoculum with buffer, the quantity of microbes was lower than in true rumen fluid. It has been found that the extent of dilution of rumen fluid with buffer for incubation modified the composition of VFAs produced (Mutzel *et al.* 2006). Another possibility is that the proportion of secondary microbial fermenters which are able to use the byproducts (such as VFA) produced by primary fermenters had increased after 12 h.

A third possibility is that the incubation system used had relatively low numbers of micro-organisms capable of degrading non-structural carbohydrates in the plant samples. As most forages have little or no starch, the fermentation of sugars would have been responsible for the early gas production and numbers of sugar-degrading micro-organisms would have increased. Later, the gas production would have been largely dependent on degradation of structural carbohydrates (Gosselink *et al.* 2004b).

Gosselink and Poncet (2002) found that ruminal outflow rate has an effect on the yield of microbial protein *in vivo* but they indicated that because there is no outflow pathway *in vitro,* microbial protein synthesis is not an ideal indicator of duodenal flow of microbial protein *in vivo*.

The *in situ* technique is probably the most widely used technique for predicting feed degradability in the rumen (Broderick and Cochran 1999). Estimates of fresh lucerne and ryegrass degradability achieved by this technique showed that effective degradability of DM and N (when outflow is assumed to be 5%/h) was higher for lucerne than for ryegrass samples whereas the potential degradability was higher for ryegrass than lucerne. These results reflected the results obtained by the *in vitro* method (even though the differences were not so clearly shown).

The method of sample preparation led to significant differences in degradation parameters, irrespective of forage species (lucerne *v.* ryegrass). The higher effective degradabilities of DM and CP of fresh *v.* frozen-thawed forages in the rumen indicates there was more intensive microbial digestion of fresh material and that freezing has a disruptive effect on plant cell structure. Therefore results obtained with this technique have to be interpreted with care.

Comparison of the results achieved from both the *in vitro* and *in situ* experiments showed that 12 h after start of the incubation OM degradation was similar for lucerne and ryegrass samples (about 50%) but it was higher at 12 h for lucerne when incubated *in situ* (60% *v.* 50%, respectively). Therefore, longer incubation times *in vitro* may provide better estimates of degradability of fresh forages. When comparing hay and silage samples (but not fresh forages), Hoffman *et al.* (2003) concluded that 16 or 24 h NDF digestibility measurements had a much higher laboratory error rate than 30 or 48 h incubations.

7.2 Rumen utilisation and abomasal flow of fresh ryegrass N

Rumen N kinetics was studied *in vivo* (see Chapter 6) by supplying ^{15}N-ammonia or ^{15}N-labelled ryegrass into the rumen. When the same quantity of ^{15}N was dosed into the rumen, the rumen ammonia-N enrichment was always lower than when ^{15}N-ryegrass was ingested. This finding indicates that ^{15}N in ingested ryegrass was not completely degraded to ammonia in the rumen. This provides further evidence that production of ammonia is not an ideal indicator of fresh forage protein

degradability *in vivo*. However, there is separate evidence that ryegrass protein was highly degradable in the rumen because the ^{15}N from ryegrass protein was almost completely removed from the whole rumen contents at the end of the first day. This finding supports the results for fresh forage samples in both the *in vitro* and *in situ* experiments.

The proportion of bacterial-N synthesized from ammonia-N (based on the relative areas under the enrichment v. time curves, (b)

Figure 6-4 was 34% when ^{15}N-ammonia was administered into the rumen (and the percentage would have been similar when ryegrass was ingested), suggesting that 66% of the bacterial-N was synthesized from NAN (*i.e.* peptides and amino acids). These values suggest that when peptides and amino acids are available in relatively high concentrations in rumen fluid, microbes are able to assimilate and use these products for their protein synthesis.

More direct evidence for the microbial utilisation of plant N in NAN in this experiment should have been obtainable from the results when ^{15}N-labelled ryegrass was fed to the animals, but an analysis similar to that used for the ^{15}N-ammonia experiment was not possible because, although the area under the microbial N pool was determined, there was no sampled primary pool (i.e. pool representing the site of administration of ^{15}N) to enable the transfer quotient to be calculated. However, the area under the microbial N v. time curve after the sheep ingested the ryegrass (1 mmol ^{15}N) was higher than when they were dosed with ^{15}N-ammonia (1 mmol ^{15}N), confirming that peptides and AA were used extensively by micro-organisms in addition to ammonia. A more complete understanding of the direct uptake of peptides and AA was obtained in Chapter 6 (Figure 6-7) by using a modeling approach (deconvolution) to remove the flux of ryegrass ^{15}N to micro-organisms via ammonia. This approach provided evidence, in support of the ^{15}N-ammonia experiment, that peptides and AA were extensively used by rumen micro-organisms. There was also further evidence that the micro-organisms assimilated much more ^{15}N than passed through both the peptide/AA and ammonia pools in rumen fluid.

A model, similar to one described by Nolan (1975) for sheep given 800 g/d of chopped lucerne hay was modified to show the potential N transactions when fresh ryegrass protein is degraded in the rumen (Figure 7-1). The pool sizes for individual pools except for bacterial N and the flux rates between pools were obtained from Chapter 6. The size of the bacterial pool used was taken from a study by Rodriguez *et al.* (2003).

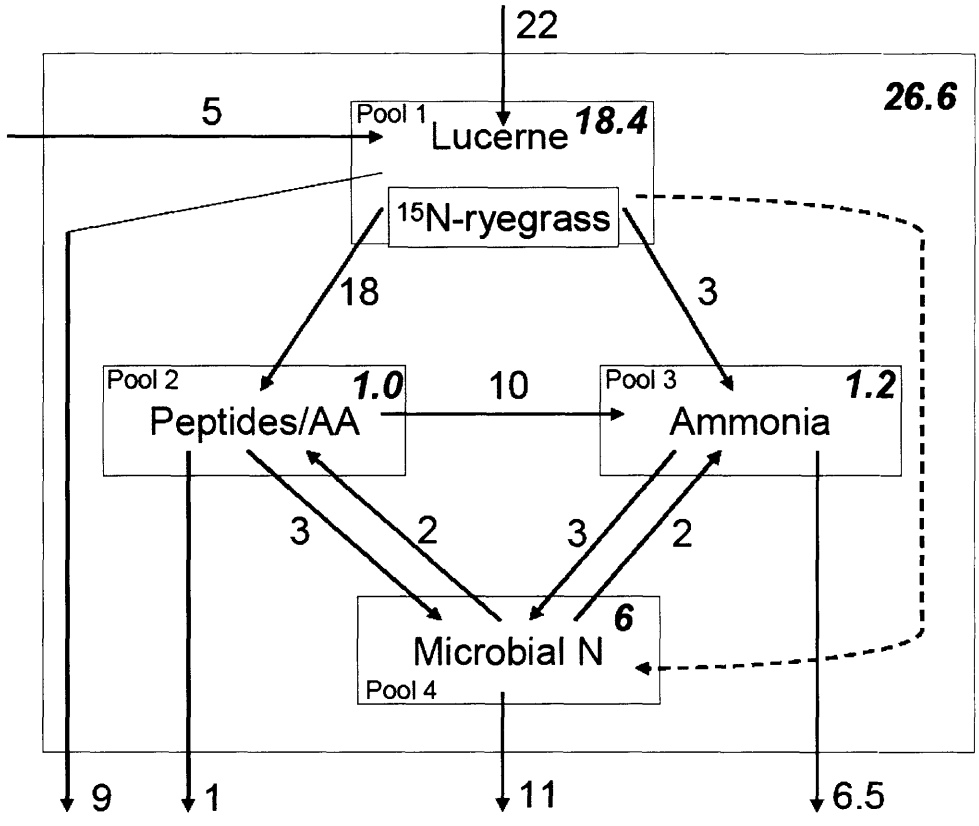


Figure 7-1. Proposed 4-pool model for N transactions in the rumen of sheep ingesting ^{15}N-labelled ryegrass (1 mmol ^{15}N) and chopped lucerne hay. The diagram shows the degradation of ryegrass and lucerne to peptides and amino acids and their utilization for microbial cell synthesis as well as the exchanges of N between these pools. Values next to arrows refer to flux rates (g N/d) and the sizes of Pools 1 to 4 are 18.4, 1.0, 1.19 and 6.0 g N, respectively.

First, this model was used to simulate the enrichment v. time curves in Pools 2, 3 and 4 in response to an injection of 1 mmol of $^{15}NH_3$ into Pool 3. The predicted

enrichment v. time curves were similar to those calculated ones in Appendix 5. Second, the model was used to simulate the enrichment curves when all the ^{15}N ingested as ryegrass (1 mmol ^{15}N) and released from Pool 1 over 1 day (assuming this occurred at an exponentially decreasing rate). In this case, the model did not satisfactorily simulate the enrichment v. time curves for Pools 2, 3 and 4 and the simulation of the ^{15}N flowing through the rumen fluid peptide/AA pools was not sufficient to account for the rate of appearance of, or the actual level of, labelling in microbial protein, i.e. the flux rate was much slower than that found *in vivo*. It seemed necessary to postulate that another direct route of transfer of ^{15}N from labelled ryegrass to microbial protein must exist from Pool 1 to Pool 4. The model was therefore modified (this is shown as the dashed arrow in Figure 7-1). The dashed arrow between ryegrass and the bacterial pool could represent microbes attached to the ryegrass particles that assimilate some labelled peptides and AA directly from the plant, thus never entering the rumen fluid. This route would be separate from, and in addition to, any assimilation from the rumen fluid pool. The importance of peptides and AA as intermediates in the microbial degradation of protein was first recognized nearly 50 years ago by Annison and Lewis (1959) who also noted ' amino acids were mainly with the micro-organisms and were either bound on the surface or were of endogenous origin'. With the information gained since 1959 showing that dietary proteins are absorbed onto the surface of rumen bacteria before proteolysis occurs, and the evidence of direct uptake of ^{15}N-labelled plant material in this study, a clearer picture of how bacterial protein degradation occurs is emerging.

Factors that may affect the relative uptake of ammonia-N or NAN for bacterial protein synthesis have been given in Chapter 6, and it is possible that the hourly feeding may have been responsible for the high ratio of NAN to ammonia utilisation for bacterial synthesis in this study. More frequent feeding, leading to a more constant release of peptides and AA through the degradation of dietary protein, may lead to a more continuous availability of these substances for microbial incorporation and an increase in the efficiency of dietary protein conversion into nitrogenous products. On the other hand, it is possible that in ruminants that are given feed once each day, ammonia may be a relatively more important source of bacterial-N.

7.3 Future studies

The studies present in this thesis have shown that it is easy to label forages with ^{15}N by growing them in pots in a glass house and watering them with a solution containing ^{15}N. When *in vitro* and *in situ* experiments were used to determine the degradability characteristics of these forages, preparation method was found important; estimates were lower in frozen-thawed than fresh forages. For future studies it would be worth measuring the quantity of carbohydrates in samples before and after freezing so that losses of carbohydrate can be determined. To enable better tracking of the disappearance of carbohydrates, samples could be taken over several hours after transferring samples to the freezer. Further samples could be taken during the period when they are thawed to provide a clearer understanding of the changes in carbohydrates availability in frozen-thawed plant materials.

Future research on *in situ* and *in vitro* techniques should be focused on their contributions to mechanistic rumen models. These models can provide helpful information without the high costs and animal welfare issues related to *in vivo* experiments. However, validity of the techniques used instead of *in vivo* experiments is very important and will require independent and large datasets to be developed, covering a wide range of diets (Gosselink *et al.* 2004a).

Another possibility for predicting the N content and degradability of fresh forages could be the use of near-infrared reflectance spectroscopy (NIRS) (Givens and Deaville 1999). Some calibrations have been generated to enable the prediction of protein degradation for frozen and fresh forages but Waters and Givens (1992) found existing calibrations were poor due to the small number of measured samples, and validation could not be carried out. Therefore, if NIRS is to be used to predict N degradability of fresh herbages, it will be important that the calibrations are generated by a standard method of sample preparation that best reflects what happens *in vivo* (Cohen and Doyle 2001).

In the *in vivo* study reported in Chapter 6, ^{15}N-labelled ryegrass was used for studying the degradation of fresh plant-N in the rumen after its ingestion by sheep

and its subsequent utilisation by rumen microbes. It would be possible to extend the results reported in this thesis by conducting a similar study using ^{15}N-labelled frozen-thawed materials as well as fresh forages. The outcomes might be different to those reported in Chapter 4 and 5. For this to be done, more ryegrass would need to be grown in pots to provide sufficient ^{15}N-labelled ryegrass to provide a complete diet for the experimental animals. This diet could then be offered to sheep on a continuous basis so that modeling of N kinetics in the rumen could be based on plateau enrichments rather than on areas under isotope decay curves such as those obtained from a single dose experiment as described in Chapter 6. It is also proposed that fresh or dried ryegrass could be fed to animals as a basal diet rather than lucerne hay as was used in the present study.

It is clear that allowing ingesting labelled plant material *in vivo* offers the prospect of obtaining more relevant information about the processes of protein degradation by grazing animals than is possible with *in vitro* methods. The most appropriate approach migh be labelling, isolating and studying kinetics of degradation of individual plant N fractions. Additional studies of lucerne degradation in sheep have already been made during the period of my candidature that are still being analysed. Information on the rate of degradation of soluble and particle associated proteins, for example, will be obtained from these experiments in due course in addition of the type of information reported for ryegrass in Chapter 6 of this thesis.

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Appendices

Appendix 1. Anthrone Methods for Carbohydrate Analysis

This method utilizes an anthrone reagent for the estimation of water-soluble or total carbohydrates in plant material. Anthrone methods have generally been displaced by phenol-sulphuric or more specific methods (Avgard 1990). The use of non-specific condensation methods has also decreased in food chemistry and human nutrition in favor of specific assays for sugars based on separation by GC or LC or on specific enzymes. However, anthrone methods have remained popular in forage analysis, but there are many variations of the method and the colour yield from specific monosaccharides is often not known for the modifications used.

Food chemists abandoned the use of anthrone in concentrated sulphuric acid as the solution is unstable and must be freshly prepared if high and variable blanks are to be avoided (see Southgate 1991). Re-crystallization of the anthrone improves this variability but not the need to always work with freshly prepared reagent solutions (re-crystallization from a 3:1 mixture of benzene and petroleum ether (60-80 bp.) or successively from benzene then ethanol, followed by vacuum drying is recommended (see Perrin *et al.* 1980).

Stable solutions in 66% (v/v) sulphuric acid with the addition of thio-urea were recommended as more stable, but the carcinogenic nature of thio-urea makes these modifications hard to recommend. The use of a 2% w/v stock solution of anthrone in ethyl acetate and the separate addition of concentrated sulphuric acid

(Avigad 1990) solves the problem of unstable stock solutions but results in a precipitate forming during the reaction that must be dissolved by vigorous mixing.

References:

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Appendix 2. *In vitro* experiment

Rumen Stoichiometry

0.5 Glucose + H_2O → Acetic + CO_2 + 4 (H)

0.5 Glucose + 2H → Propionic + H_2O

Glucose → Butyric + $2CO_2$ + 4 (H)

Glucose + 2H → Valeric + CO_2 + 2 H_2O

Table 7-1. Concentration of volarile fatty acids in the incubation bottles containing rumen fluid and buffer only (blanks)

Sample	Replicate	Incubation time	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
Blank	1	0	18.81	4.38	0.30	3.70	0.43	0.31
Blank	2	0	17.57	4.39	0.23	3.56	0.45	0.29
Blank	1	3	17.56	4.89	0.31	4.27	0.51	0.34
Blank	2	3	21.18	5.34	0.34	4.62	0.53	0.36
Blank	1	6	20.00	4.36	0.27	3.80	0.53	0.32
Blank	2	6	19.79	4.97	0.33	4.40	0.62	0.36
Blank	1	9	20.64	4.46	0.41	4.11	0.67	0.36
Blank	2	9	20.91	5.26	0.48	4.66	0.70	0.40
Blank	1	12	21.46	5.83	0.60	5.34	0.92	0.49
Blank	2	12	19.45	4.98	0.50	5.51	0.78	0.43

Coefficients of fitted curves for total VFA - Figure 4-1

Nonlinear Regression - data fitted using SigmaPlot (2006)
Data Source: Data 1 in cumulative TVFA curve.
Equation: Single Exponential Rise to Maximum, 2 Parameters (a, b)

$f=a*(1-\exp(-b*x))$
where x is time

Fresh lucerne

R	R^2	Adj R^2	Standard Error of Estimate
0.9812	0.9627	0.9503	0.4402

	Coefficient	Std. Error	t	P	VIF
a	6.0211	0.9169	6.5669	0.0072	8.9617
b	0.1831	0.0638	2.8721	0.0639	8.9617

Frozen-thawed lucerne

R	R^2	Adj R^2	Standard Error of Estimate
0.9958	0.9917	0.9889	0.1653

	Coefficient	Std. Error	t	P	VIF
a	4.8000	0.3589	13.3731	0.0009	9.5245
b	0.1783	0.0299	5.9555	0.0095	9.5245

Frozen-thawed lucerne + starch

R	R^2	Adj R^2	Standard Error of Estimate
0.9927	0.9854	0.9806	0.2402

	Coefficient	Std. Error	t	P	VIF
a	4.8205	0.3521	13.6912	0.0008	5.3176
b	0.2335	0.0463	5.0411	0.0151	5.3176

Fresh ryegrass

R	R^2	Adj R^2	Standard Error of Estimate
0.9855	0.9713	0.9617	0.3276

	Coefficient	Std. Error	t	P	VIF
a	5.5481	1.2023	4.6145	0.0191	20.1085
b	0.1297	0.0519	2.4968	0.0880	20.1085

Frozen-thawed ryegrass

R	R^2	Adj R^2	Standard Error of Estimate
0.9769	0.9543	0.9390	0.3581

	Coefficient	Std. Error	t	P	VIF
a	6.2229	2.8956	2.1491	0.1208	57.7418
b	0.0834	0.0583	1.4300	0.2481	57.7418

Frozen-thawed ryegrass + starch

R	R^2	Adj R^2	Standard Error of Estimate
0.9891	0.9782	0.9710	0.2375

	Coefficient	Std. Error	t	P	VIF
a	4.2879	0.5210	8.2309	0.0038	9.6650
b	0.1771	0.0481	3.6812	0.0347	9.6650

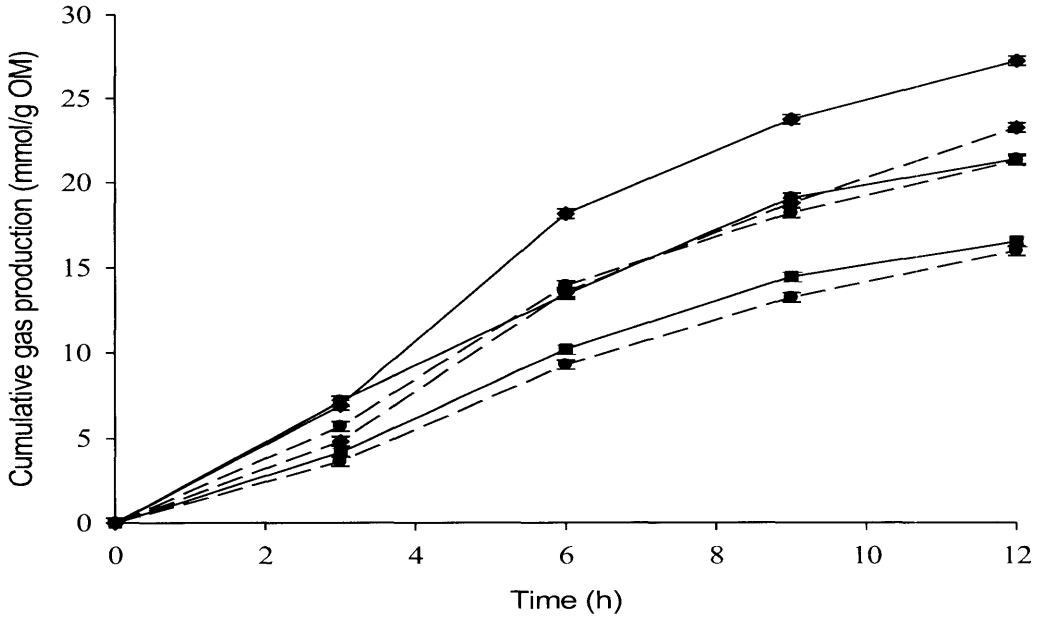


Figure 1-A. Cumulative in vitro gas production (mmol/g OM per 3h) in lucerne (—) and ryegrass (---) for each method of sample preparation: fresh (♦), frozen-thawed (■) and frozen-thawed + starch (●) at different incubation sampling times (Means ± SEM).

Appendix 3. Accuracy and repeatability of the ^{15}N analysis

Repeatability of ^{15}N analysis was inspected by including one standard (Standard1, wheat flour with known enrichment of 0.3650 mol ^{15}N/mol N) in every run of ^{15}N analysis (usually in 50-100 analyses done on one day). Another standard (Standard 2, $(NH_4)_2SO_4$ with known enrichment of 0.539%) was also used. ^{15}N abundance analyses for the standards obtained on various occasions are presented in Table 1- .

Table 1-A. Representative results of ^{15}N abundance analyses for standards

Standard 1		Standard 2	
Date	^{15}N Abundance (%)	Date	^{15}N Abundance (%)
14/12/2005	0.3659	2/05/06	0.5435
4/01/2006	0.3650	2/05/06	0.5364
19/01/2006	0.3656	22/06/06	0.5376
17/03/2006	0.3650	25/09/06	0.5424
28/03/2006	0.3652		
28/03/2006	0.3650		
18/04/2006	0.3653		
2/05/2006	0.3650		
9/06/2006	0.3650		
22/06/2006	0.3650		
24/08/2006	0.3658		
Mean	0.3653		0.5400
CV	0.00024		0.00557

Appendix 4. Recovery of Cr standards in Cr analysis

In the earlier stages of Cr analysis on the ICP-OES, recovery of standards containing a known amount of Cr was inspected. Those standards were spiked into samples of fluid or particle-rich abomasum digesta containing no extraneous Cr. Details of digestion process are given in Section 3.2.3. Representative recovery data are presented in Table 7-. It was concluded that the Cr analyses was accurate with a mean percentage recovery 92.9 and a standard deviation of 1.2%.

Table 7-A Recovery of Co standards analysed on an ICP-OES

Cr concentration ug/ml		
Calculated	Measured	Recovery (%)
4.019	3.783	94.1
4.462	4.121	92.4
3.978	3.754	94.4
4.017	3.714	92.5
4.047	3.690	91.2
4.009	3.715	92.7
	Mean	92.9
	CV	1.5

Appendix 5. Simulated models

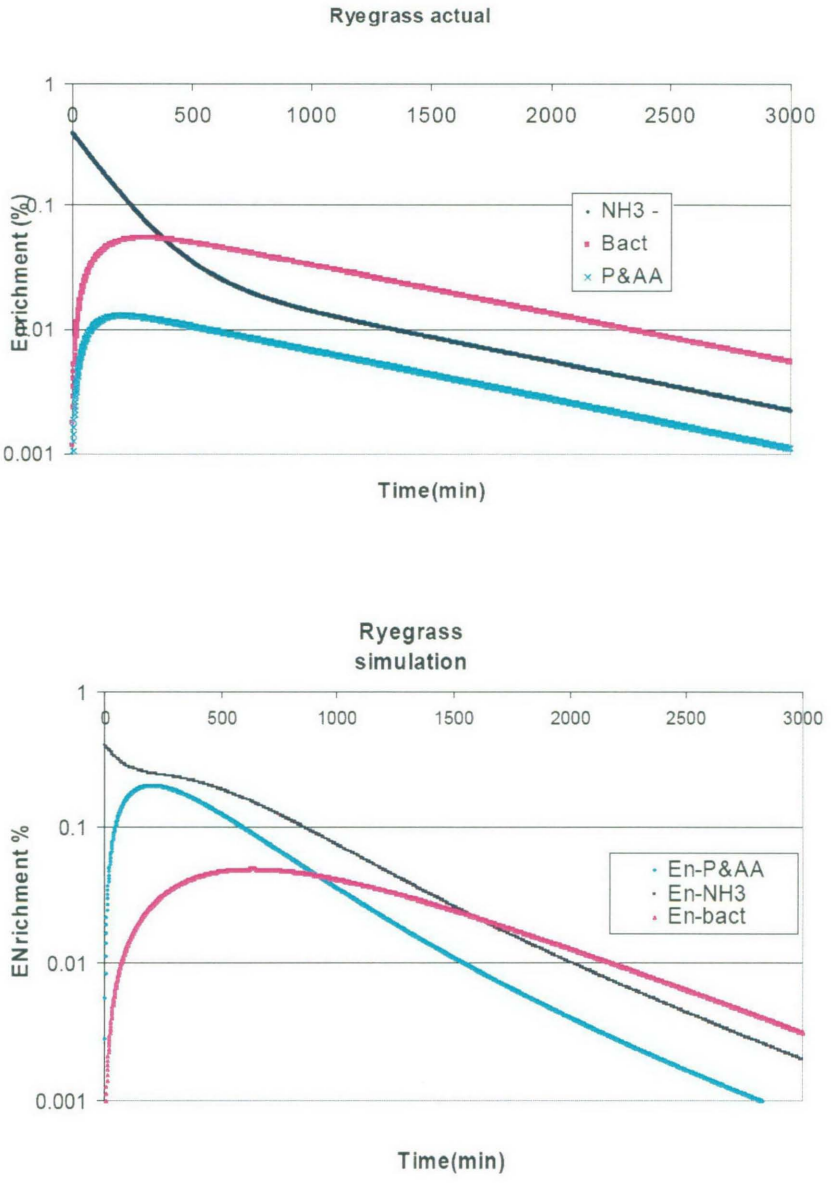


Figure 2-A. Enrichment curves (actual (top) and simulated (bottom)) of NH_3, bacteria and peptides and amino acids when animals fed ^{15}N labelled ryegrass.

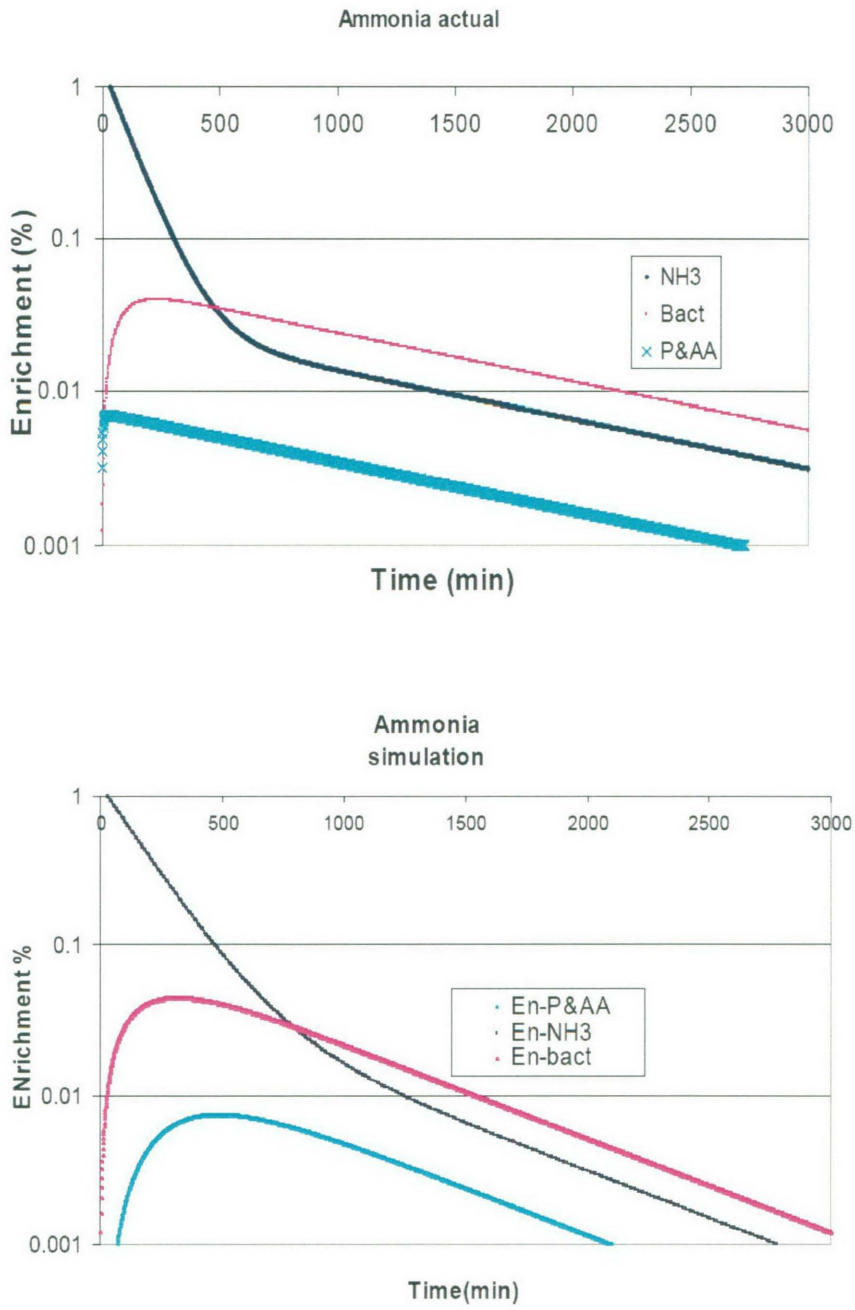
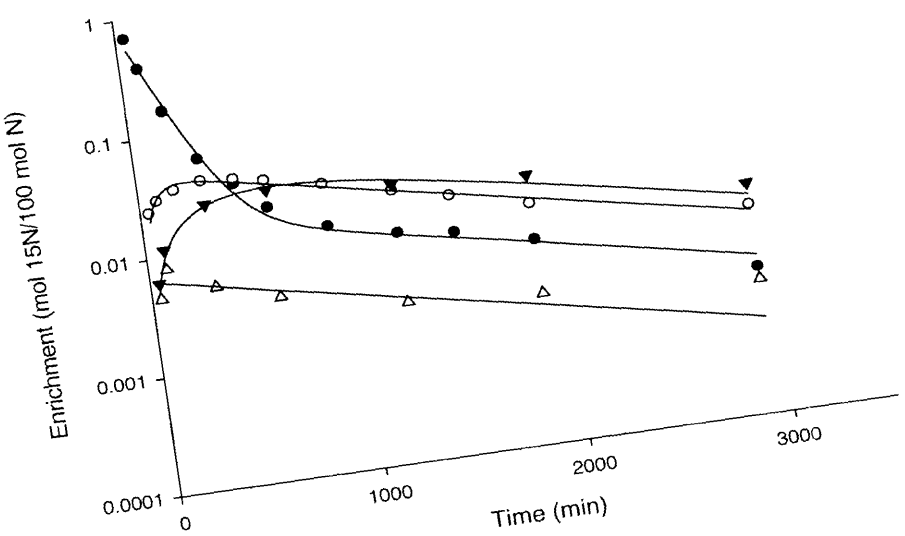


Figure 3-A. Enrichment curves (actual (top) and simulated (bottom)) of NH_3, bacteria and peptides and amino acids when animals fed ^{15}N ammonia.



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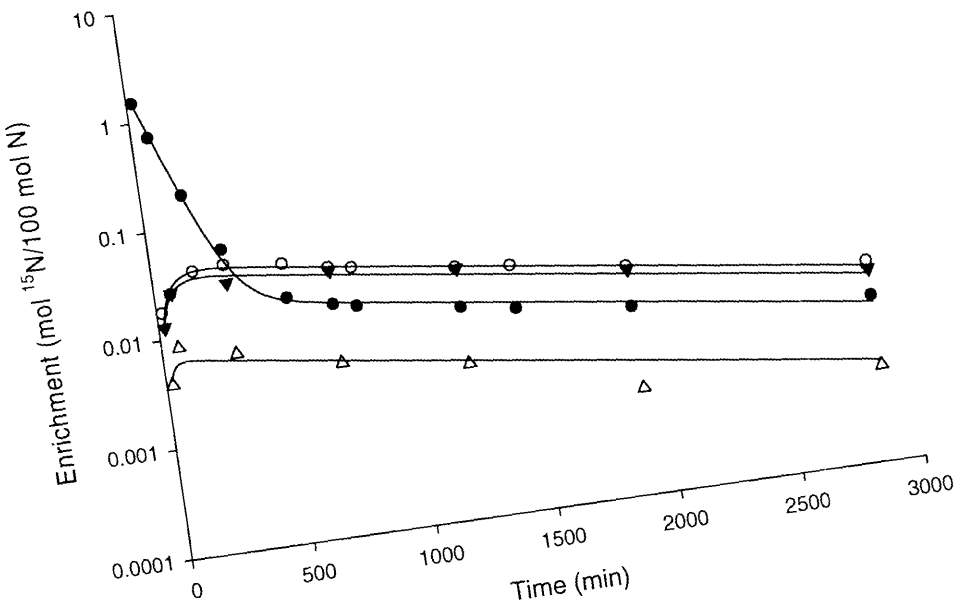

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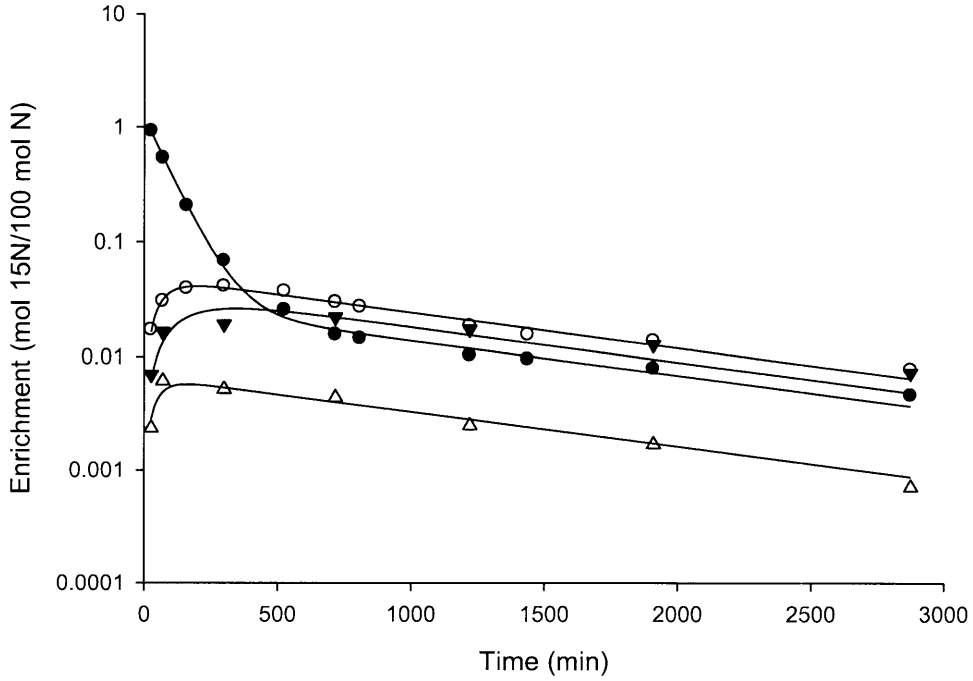

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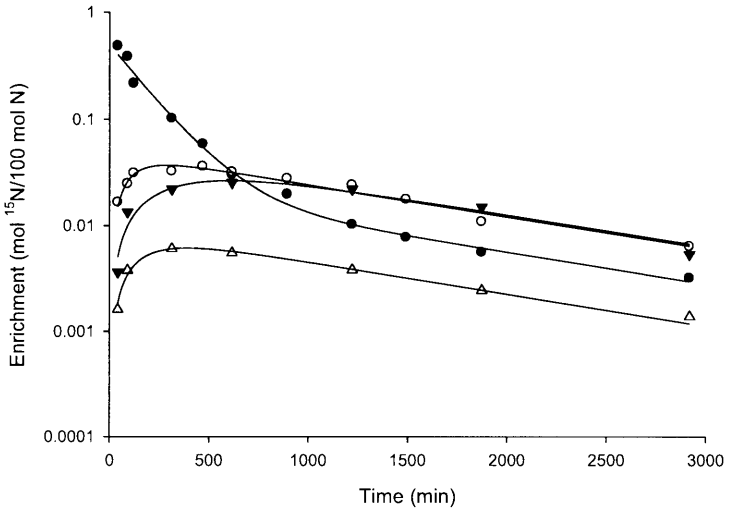

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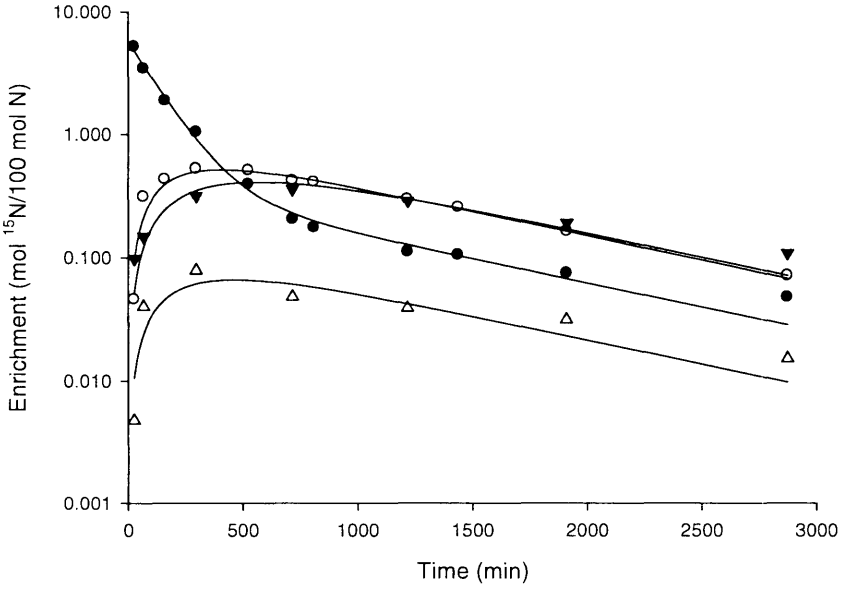
Figure 4-A. Enrichments (y axis; mol ^{15}N/100 mol total-N) in rumen fluid ammonia (•), rumen fluid bacterial-N(○), peptides and amino acids (⌂) and soluble

proteins (▼) versus time (x axis; min) after intra-ruminal administration of 1 mmol ^{15}N as ^{15}N-labelled ammonia



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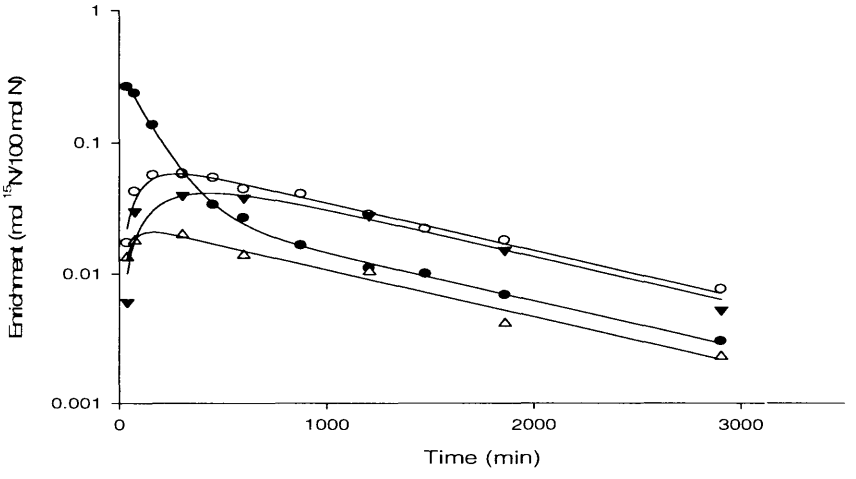

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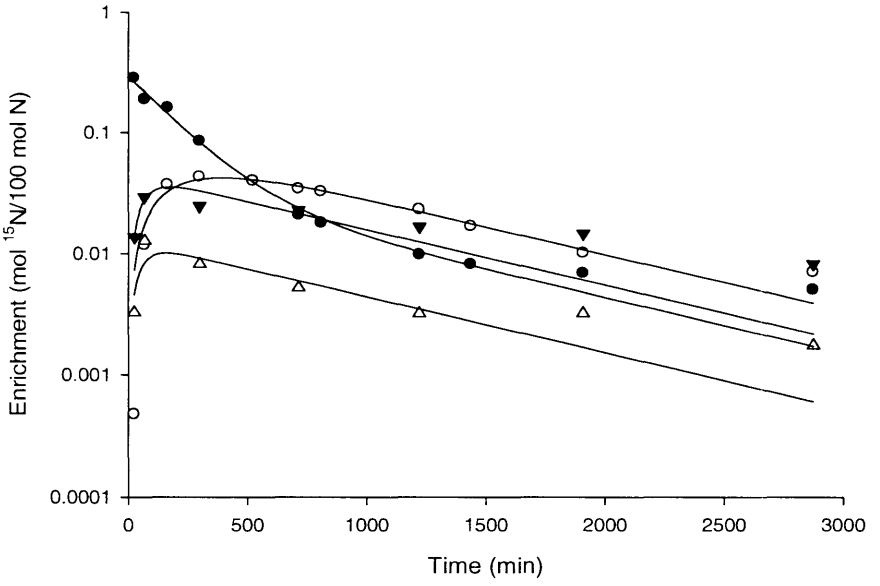

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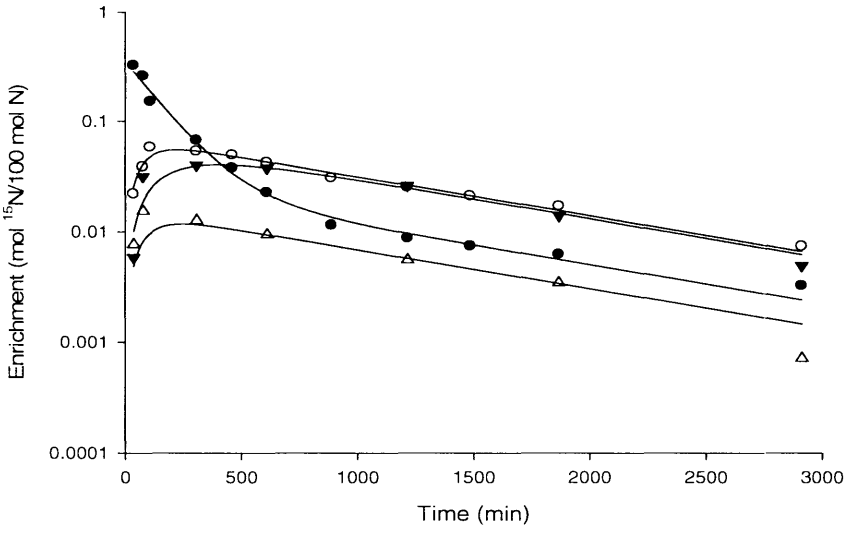

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Figure 4-B. Enrichments (y axis; mol ^{15}N/100 mol total-N) in rumen fluid ammonia (•), rumen fluid bacterial-N(○), peptides and amino acids (⌂) and soluble proteins (▼) versus time (x axis; min) after intra-ruminal administration of 1 mmol ^{15}N as ^{15}N-labelled ryegrass