ABSTRACT

Title of Thesis: REGULATION OF UREA RECYCLING INTO THE GASTROINTESTINAL TRACT AND AMMONIA METABOLISM IN RUMINANTS

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The primary objective of this study was to determine the extent ruminants control urea recycling to the gastrointestinal tract (GIT), independent of rumen microbial metabolism. Urea kinetics were determined by continuous infusion of [$^{15}N^{15}N$]urea (4 levels) to jugular vein of sheep (n = 4; 28.1kg) fed a low protein (6.8%) diet. Although urea recycling to the GIT ($P < 0.001$) increased with each level of urea, the proportion ($P < 0.001$) and amount ($P < 0.001$) of recycled urea returning as ammonia to liver for ureagenesis also increased. In consequence a decreasing proportion ($P = 0.003$) of recycled urea was used for microbial protein synthesis. The present study suggests that ruminants have a large capacity to partition urea-N to the GIT. Thus, rather than up-regulating urea recycling, there appears to be more potential to improve N efficiency in ruminants by manipulating the rumen environment to optimize capture of recycled N.

REGULATION OF UREA RECYCLING INTO THE GASTROINTESTINAL TRACT AND AMMONIA METABOLISM IN RUMINANTS

By

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1.1 Introduction

Ruminant animals convert only 5 to 35% of dietary N into salable products. The two main areas where significant losses of N occur are: 1) the intense metabolism of the GIT, for example only 0.25 - 0.6 of essential amino acids disappearing from the small intestines is recovered in the portal vein. 2) Ammonia absorption (46 - 47% of N available in the lumen of gut) and urea excretion into urine (30 – 70% of urea production). This project considers one aspect of urea recycling and ammonia metabolism in ruminants. Through a better understanding of urea recycling, it may be possible to enhance performance (efficiency) of animals and reduce N losses to the environment while at the same time reduce feed costs associated with feed supplements.

Urea is the major end product of NH_3 and amino acid metabolism in animals and is produced by the liver in greater amounts than what is excreted in the urine. Urea not excreted into the urine has two fates: 1) Partition to the rumen where it is hydrolyzed to NH_3 and then either reabsorbed into the blood as ammonia or incorporated into microbial protein for intestinal absorption and partition to the lower gut (cecum, large intestines) and loss as fecal microbial N. Gut urea recycling occurs in mammals (10-80%) and to some extent in poultry (by transport of urine from cloaca into the colon and ceca by retrograde peristalsis) but in ruminants the proportion of urea entering the gut is much higher and of greatest importance to N economy of the ruminant. In ruminants, values have been reported in the range of 40-80% of urea produced by the liver partitioned to the GIT. Urea is recycled to all sections of the GIT with, on average,

roughly equal amounts recycled to the rumen and small intestines. However, for recycled urea to contribute to absorbable microbial protein, and thus amino acid absorption, urea must be recycled to and captured in the rumen. Typical values for the partition of urea between urine and GIT are 60:40 to 20:80, depending upon type of diet and level of feed and protein intake.

A primary goal of ruminant nutritionists is to reduce urinary and fecal nitrogen excretion and pollution of the environment, and in this respect increasing the partition of blood urea to the rumen rather than into the urine and increasing the capture of ammonia in the rumen by microbes, is a means to accomplishing this goal.

To date, our knowledge of the mechanisms regulating urea partition to the GIT of ruminants is limited. For example, while it has been observed to that higher levels of food and protein intake enhance urea recycling to the GIT, the exact mechanisms involved are largely unknown. However, recent observations that the rumen tissues express a urea transporter suggests that this process is probably regulated and that it may also involve humoral factors (e.g. hormones, substrate concentration). This suggests that in addition to events occurring within the rumen environment, the animal itself may have some control over urea recycling. If the latter can be demonstrated, then it may be possible to enhance this process and mechanisms by feeding, genetics or other consumer acceptable means to enhance urea recycling and rumen capture; thus reducing urine losses of nitrogen and improving the efficiency of feed nitrogen utilisation in growing and lactating ruminants.

The aim of this thesis is to determine the extent and some general mechanisms by which the ruminant animal, independent of metabolism in the rumen (i.e.

fermentation rate, microbial activity, rumen ammonia level), control the process of urea partition to the GIT.

1.2 Overview of Urea and Ammonia Metabolism

Nitrogen that is consumed by ruminants is in various forms such as nucleic acids, amino acids, proteins, peptides, amines, amides, nitrates, nitrites, urea, ammonia and endogenous sources (sloughed cells and recycled urea). Most of these N sources are readily degraded in the rumen and the microbial organisms utilize the ammonia produced for microbial protein synthesis. Thus the requirement of ruminants for amino acids derive mostly from rumen microbial protein (40 -70%; Clark *et al*., 1992) and from dietary protein not degraded in the rumen. (ie. rumen bypass protein). However, ruminants are very inefficient (~20%) at converting dietary protein into tissue gain or milk secretion and wool growth. Furthermore, once amino acids are absorbed, efficiency of utilization is in the range 30-50%, much lower when compared to the 60-70% observed in pigs (MacRae *et al*., 1996). The large amounts of ammonia produced, especially in forage fed animals, and the energetic cost associated with disposal of this ammonia as urea is one of the main contributors to post-absorptive inefficiency.

Large amounts of ammonia produced as a result of rumen microbial fermentation are transported to the liver and converted to urea. Estimates are that ~50% of the total N supply to the rumen enters the rumen ammonia pool (Huntington and Archibeque, 1999). Ammonia can diffuse across all sections of GIT in ruminants. However, it is only the unprotonated ammonia that diffuses across the rumen epithelial wall into the blood, whereas the protonated form does not diffuse into the blood. Within the epithelial cell, ammonia is protonated to form ammonium ions which are then

removed by the liver (Huntington and Archibeque, 1999). Therefore, the extend of ammonia absorption is primarily a function of the ratio in the rumen fluid of unionized to ionized ammonia.

1.3 Urea Synthesis

Urea synthesis (Figure 1) plays an important role in the detoxification of absorbed ammonia, disposal of excess amino acid N and maintenance of peripheral blood concentrations optimal for reactions such as synthesis of nucleic acids (Lobley *et al*., 1995). Because urea is water soluble and ten times less toxic than ammonia, ureotelic animals can dispose off urea in lower volumes of urine than required for ammonia disposal. Ammonia produced in the GIT and peripheral tissues is transported to the liver through the portal vein where it is converted to urea via the ornithine-urea cycle (OUC). Apparent fractional extractions of 0.75-0.85 by the liver indicate that the liver is a very efficient at removing absorbed ammonia (Lobley and Milano, 1997).

There is an energetic cost and a potential N cost associated with hepatic ureagenesis. Estimates are that the energetic costs of urea synthesis account for 13-16% in cattle and 13-19% in sheep of hepatic oxygen consumption. This is based on the assumption that four high-energy phosphate bonds are broken per molecule of urea synthesised (Lobley *et al*., 1995).

Five enzymes (Figure 1) comprise the OUC and these are distributed between the cytosol and mitochondrial compartments of periportal and perivenous hepatic cells. Periportal cells remove most of the ammonia from the hepatic portal blood and convert it to urea. Perivenous cells have high glutamine synthetase activity, these cells act as a sink to eliminate the excess ammonia not removed by the periportal cells. Ammonia is

converted to glutamine before the blood enters the hepatic vein and subsequently the systemic circulation (Haussinger, 1990). Urea cycle enzymes are present not only in hepatocytes, but also in small intestinal enterocytes (Wu, 1995). Oba *et al*., (2004) demonstrated that urea could be synthesised by rumen epithelial and duodenal mucosal cells in vitro, although the regulation of this pathway in these tissues may differ from that in the liver.

Hepatic ureagenesis requires the coordinated supply of N to the ornithine cycle from mitochondrial NH_3 and cytosolic aspartate (Huntington, 1989; Reynolds *et al*., 1991; Lobley *et al*., 1995). The mitochondrial ammonia is generated predominantly by the action of glutaminase, which channels ammonia directly to carbamoyl phosphate synthetase. A second mechanism involves glutamate dehydrogenase which provides ammonia to aspartate via transamination reactions with glutamate.

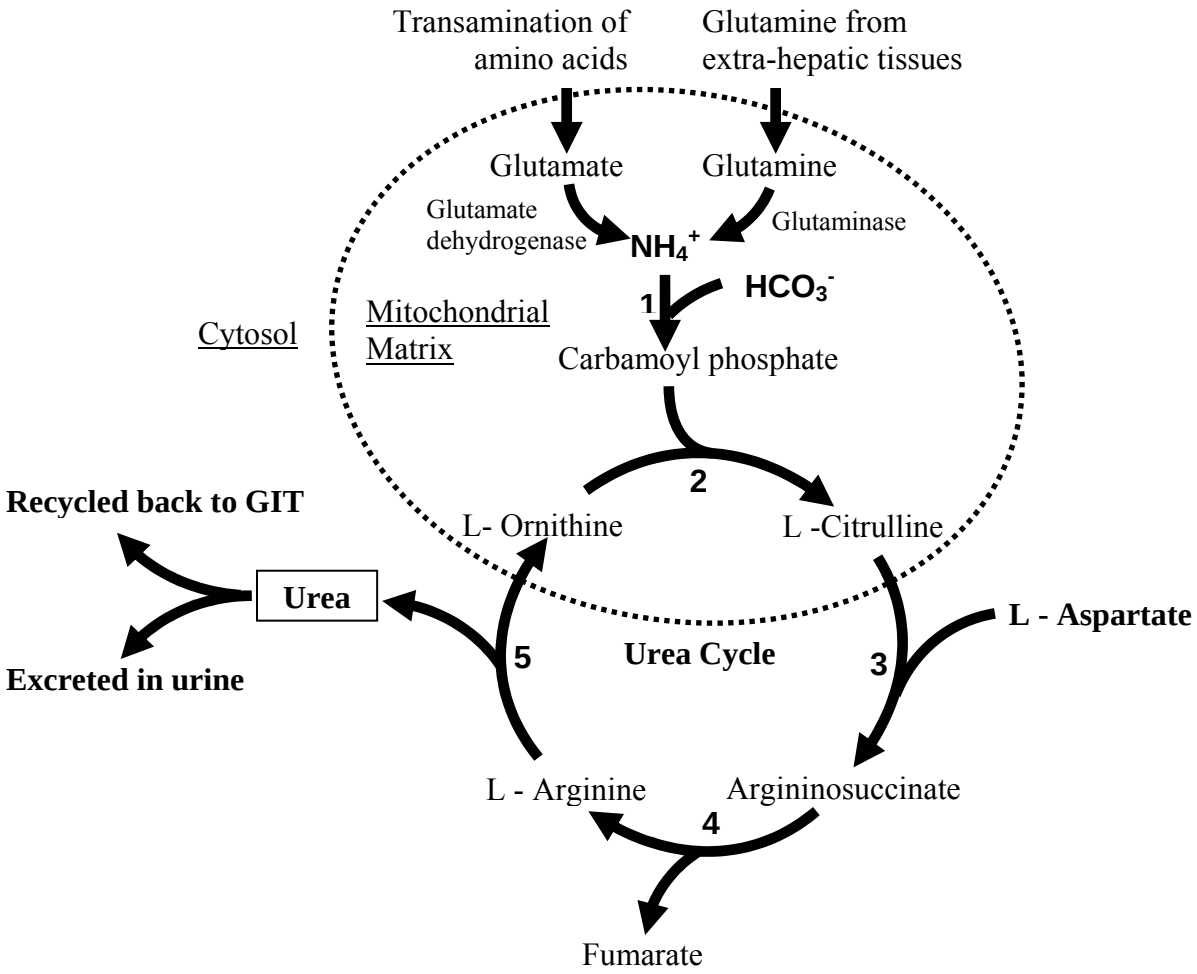


Figure 1. Reactions and intermediates of urea biosynthesis. Mitochondrial NH_4^+ and cytosolic aspartate provide the two N atoms for urea synthesis. Five enzymes are involved in urea cycle. **1**. Carbamoyl phosphate synthase I, **2**. Ornithine transcarbamoylase, **3**. Argininosuccinate synthase, **4**. Argininosuccinate lyase, **5**. Agrinase.

1.4 Effect of Ureagenesis on Amino Acid Economy

One of the main concerns about high ammonia concentrations was whether it compromises the amino acid economy of the animal. Free amino acids from the blood and ammonia are the main N substrates extracted by the liver for maintaining the rate of ureagenesis (Huntington, 1989; Reynolds *et al*., 1991; Lobley *et al*., 1995). This led to the hypothesis that urea synthesis requires equal inputs of N from both ammonia and free amino acids, thus potentially having a negative effect on the amino acid economy (eg. essential amino acids). For example, Lobley *et al.,* (1995) observed a two-fold higher rate of hepatic urea-N output for each unit of ammonia N removed by the liver. Concurrently they also observed an increase in leucine oxidation suggesting that essential amino acids might be catabolised to provide the additional N input at aspartate. However, in later studies by this group (Lobley *et al.*,1995) with isolated sheep hepatocytes in vitro, they clearly showed that the N for both aspartate and carbamoyl phosphate can arise from ammonia. Here 90% of urea was as [^{15}N ^{15}N] urea when hepatocytes were incubated with [^{15}N] ammonia. These results were substantiated by Milano et al., (2000), where increasing levels of ammonium bi carbonate infused into the mesenteric vein did not alter net amino acid supply to peripheral tissues. Hence, a linear relation-ship was observed between hepatic urea-N release and liver ammonia extraction (r^2 0.89), with the slope of regression, (1.16) not different from unity.

1.5 Urea Recycling

Nitrogen transfers across the GIT can be much greater than the N intake and thus play an important role in the N metabolism of the ruminant. Even in ruminants fed high food intakes, urea synthesis exceeds apparent digestible N intake resulting in negative N balance if the animal does not recycle some of the urea synthesized in the liver (Lapierre and Lobley, 2001). Urea is distributed throughout all body fluids and enters all compartments of the GIT through secretions (i.e. saliva, gastric juice, bile and pancreatic juice) and by diffusion from blood (Kennedy and Milligan, 1980; Egan *et al*., 1984). Recycling of urea back to the GIT occurs in all animals (Table 1) though the magnitude of recycling is much greater in ruminants (10-80% of urea synthesis) than in non-ruminants. Thus, urea recycling becomes the main N conservation mechanism especially in high producing and rapidly growing ruminants, in particular when N supply is low (eg. at maintenance intake levels). Ruminants rely upon the presence of a large and active microbial population in the rumen, to ensure that recycled urea-N (via rumen wall and saliva) is utilized for microbial protein synthesis and absorption. A number of studies have quantified the GIT entry of urea (Kennedy and Milligan, 1978; Benlamlih and Pomyers, 1989; Mosenthin *et al*., 1992; Archibeque, *et al*., 2001) however, only a few of these studies have estimated the anabolic fates of recycled urea (Sarraseca, *et al*., 1998; Lobley, *et al*., 2000; Marini and Van Amburgh, 2003)

Table 1. Urea recycling in different species of animals (g N/day)

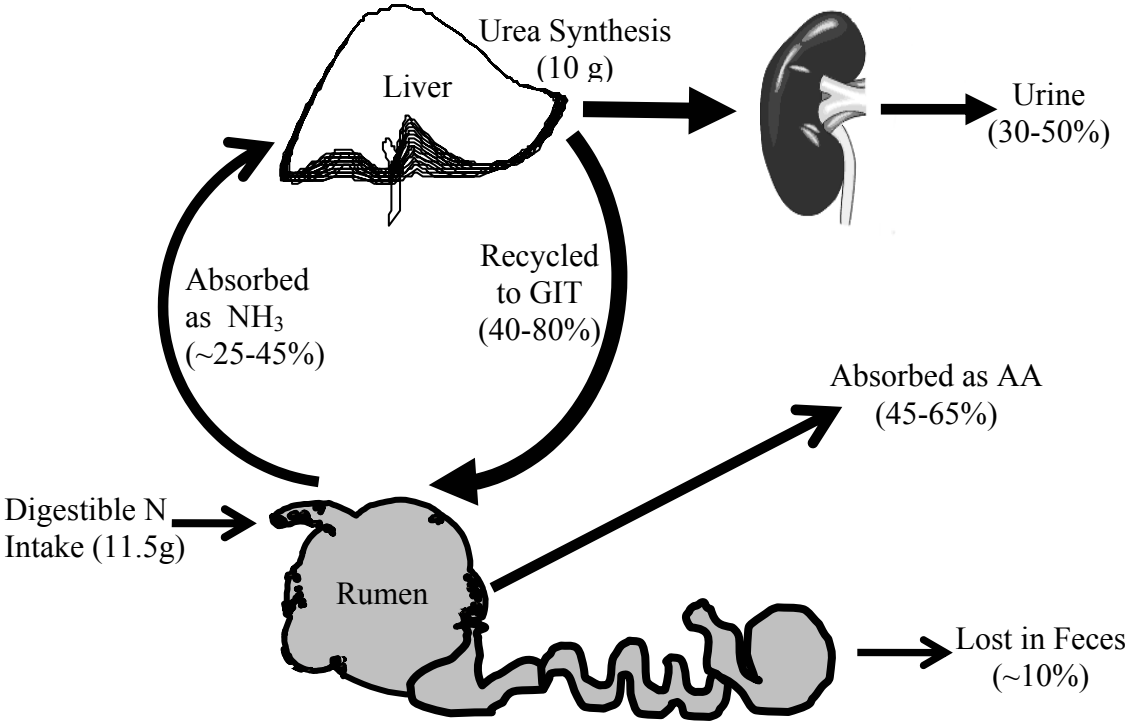
	N intake	Digestible N	Urea-N synthesis	Urea-N recycled to GIT	**Gut return: Synthesis**
Dairy cows	450	301	262	171	**0.67**
Steers[2]	64	33.1	35.4	28.1	**0.79**
Sheep[3]	17.1	11.5	16.3	9.9	**0.61**
Human[4]	10.3	--	11.3	4.4	**0.39**
Cats[5]	1.7	1.5	1.1	0.2	**0.15**
Pigs[6]	28.1	18.8	21	9.7	**0.46**

[1]Lapierre et al., Unpublished
[2]Archibeque et al., 2000
[3]Lobley et al., 2000
[4]McClelland and Jackson, 1996
[5]Russell et al., 2000
[6]Adapted from Mosenthin *et al*., 1992.

1.6 Fates of Urea Entering the GIT

Most of the urea entering the GIT is hydrolyzed to NH_3 by the action of bacterial urease. The released ammonia is either reabsorbed as ammonia (~40%) or utilized as a N source for microbial protein synthesis (~50%) with the remaining getting excreted in the feces (~10%; Figure 2). The NH_3 reabsorbed from the GIT can be converted to citrulline by the GIT tissues or converted to urea by the liver. Similarly, the amino acids of bacterial origin, derived from recycled urea-N, may also be absorbed and catabolized by the liver to yield urea (Sarraseca *et al.*, 1998; Milano *et al*., 2000), and the cycle continues. The fractional contributions of recycled urea-N towards each of these fates depends upon various conditions in the rumen (pH, ammonia/ammonium concentration, fermentable energy) and also dietary factors such as protein content, concentrate/forage diet, available or fermentable energy etc.

Recycled urea can be partitioned to different compartments of the GIT, i.e. the fore-gut (rumen), the mid-gut (small intestine) and hind-gut (large intestine). This partitioning depends on various factors such as dietary N intake, fermentability of the carbohydrate fraction of the diet etc (Kennedy and Milligan, 1980). As far as known, only the urea transferred to and hydrolyzed in the rumen for microbial protein synthesis is ultimately absorbed in the small intestines.



(Lobley *et al*., 2000)

Figure 2. Urea recycling in sheep. Of the total urea synthesised in the liver (10g), 30-50 % is excreted in the urine and 40-80 % is recycled back to the GIT. Of this portion recycled back to the GIT, 25-45 % is reabsorbed to liver as ammonia where it is reutilized for the synthesis of urea, 45-65 % is absorbed as amino acids which is utilized for productive purposes and around 10 % is excreted in feces.

1.7 Urea Transfer to the Rumen

As far as known, conversion of recycled urea-N into microbial protein for absorption can only occur from urea-N recycled to the rumen (Egan *et al*., 1984; Mosenthin *et al*., 1992; Lapierre and Lobley, 2001). Estimates in sheep are that 27-60% of gut entry is to the rumen (via saliva or rumen wall) (Kennedy and Milligan, 1978; Koenig *et al*., 2000). Kennedy and Milligan (1980) suggested that rumen clearance of urea (theoretically the same as rumen urea entry), the product of rumen epithelial permeability to urea and the functional area of rumen epithelia is greater in sheep compared to cattle. This may be due to a greater permeability of sheep rumen epithelia to urea (1.7 times than in calf) or due to difference in papillary bed (e.g. more surface area in sheep). These observations suggest that sheep may be more efficient in utilizing recycled urea for anabolic purposes compared to cattle.

How does urea cross the rumen epithelium? Wallace *et al*., (1979) proposed a hypothesis based on the urease activity in the rumen. Here, urease which penetrated the cornified ruminal epithelium rapidly broke down the urea molecules and created a concentration gradient of ammonia thereby pulling urea molecules into the more acidic rumen environment. Studies as early as 1965 also suggested that urea transport across ruminal epithelium followed saturation kinetics implying the existence of an active transport system. Recently, carrier mediated facilitative urea transport mechanisms have been identified in rumen epithelia and ovine colon, which aids in the bidirectional urea transport of urea by the tissue (Ritzhaupt *et al*., 1997; 1998; Marini and Van Amburgh, 2003).

1.8 Urea Transfer to the Small Intestines

Up to 70% of the gut entry rate (GER) has been reported to enter post stomach tissues (small intestine and large intestine) with the proportion contributing to small intestine greater (90%) in ruminants fed forages compared to concentrates (19%). However most of the urea-N entering the small intestines is converted to ammonia and returned to the liver (Reynolds and Huntington, 1988; Huntington, 1989). Observations have been made in non-ruminants where urea-^{15}N and ammonia ^{15}N delivered into the intestine or colon was found to be incorporated into lysine and threonine derived from blood and tissue proteins. As these amino acids do not undergo transamination in the body, the implications are that hind-gut microbial protein can be absorbed and contribute to amino acid nutrition of non-ruminants. However, Lapierre and Lobley (2001) suggested that the above mechanism is mediated via microbial protein synthesis and in ruminants may not be of much importance towards anabolic use of recycled N

1.9 Urea Transfer to the Cecum and Colon

Studies by Dixon and Milligan (1983) and Bergner *et al*. (1986b) suggest a minimal role for the lower digestive tract (cecum, colon) in the degradation of urea and reutilization of urea-N by hind gut microbes. They found that most of the urea-N transferred to the hind-gut appeared in the feces. Mosenthin *et al* (1992) recovered only 0.09% of the intravenously infused ^{15}N label from cecum and colon of pigs indicating a minor role for the large intestines as a site for anabolic usage of urea. Estimates in sheep are that 0.3 to 1.6 g of urea-N /day (5-10% 0f urea entry to GIT) is lost in feces via hind gut fermentation (Kennedy and Milligan, 1980), and this appears to vary widely depending on the intake, forage and concentrate diets, presence of rumen protozoa and

the presence of intestinal parasites. Provision of fermentable energy to the cecum appears to increase the proportion of urea gut entry lost to feces (10 –25%).

1.10 Salivary Transfer of Urea to the GIT

Saliva is also a significant route (15-100%) of urea-N recycling to the rumen depending on the type of diet (concentrate/forage) (Huntington, 1989). As recycling of urea through saliva can be measured as the product of saliva flow rate and urea concentration, factors affecting the flow rate, for example rumination activity, feed intake etc can affect urea transfer through saliva (Egan *et al*., 1986). For example a diet high in fiber or dry forage stimulates rumination activity which in turn increases the salivary flow to the rumen. Marini and VanAmburgh, 2003 observed an increase in salivary transfer of urea with high levels of N intake. The amount transferred were approximately 3-4% of the total urea gut entry. Generally salivary transfer dominates when the animal is fed a forage diet (around 70%) compared to a concentrate diet (around 23%; Lapierre and Lobley, 2001).

1.11 Utilization of Recycled Urea in the Rumen

Ammonia produced in the GIT from the hydrolysis of urea entering the GIT can be reutilized for synthesis of amino acid which can be reabsorbed and used for productive purposes. The percent of recycled urea used for anabolism within the rumen ranges from 46% to 63%, depending on the level of intake, type of diet, and fermentable energy intake (Sarraseca et al., 1998; Lobley et al., 2000; Archibeque et al., 2000a, 2000b). It is possible for the urea-N to reenter ornithine cycle several times without getting excreted in the urine. Such multiple entries of the same urea- N into the ornithine

cycle also increase the probability of capture and use of urea-N for anabolic purposes. Conversion of apparent digestible N to amino acid N is more efficient in sheep (81%) than in steers and dairy cows (39-55%), however the biological reasons for this are not clear. One of the reasons for this may be the high GIT entry of urea in sheep and their subsequent anabolic usage as discussed previously. It may also be related to the type of dry diets studied or the low intakes offered or the moderate growth potential of the animals used (Lapierre and Lobley, 2001).

Observations that different GIT tissues express a full complement of urea cycle enzymes (Wu, 1995; Oba, 2004) raise the possibility that the gut tissues could synthesize urea from luminal ammonia thus reducing ammonia absorption. This could be a potential target to improve local recycling of urea to the rumen and for reducing the toxic effects of blood ammonia on post absorptive tissue (Oba, 2004).

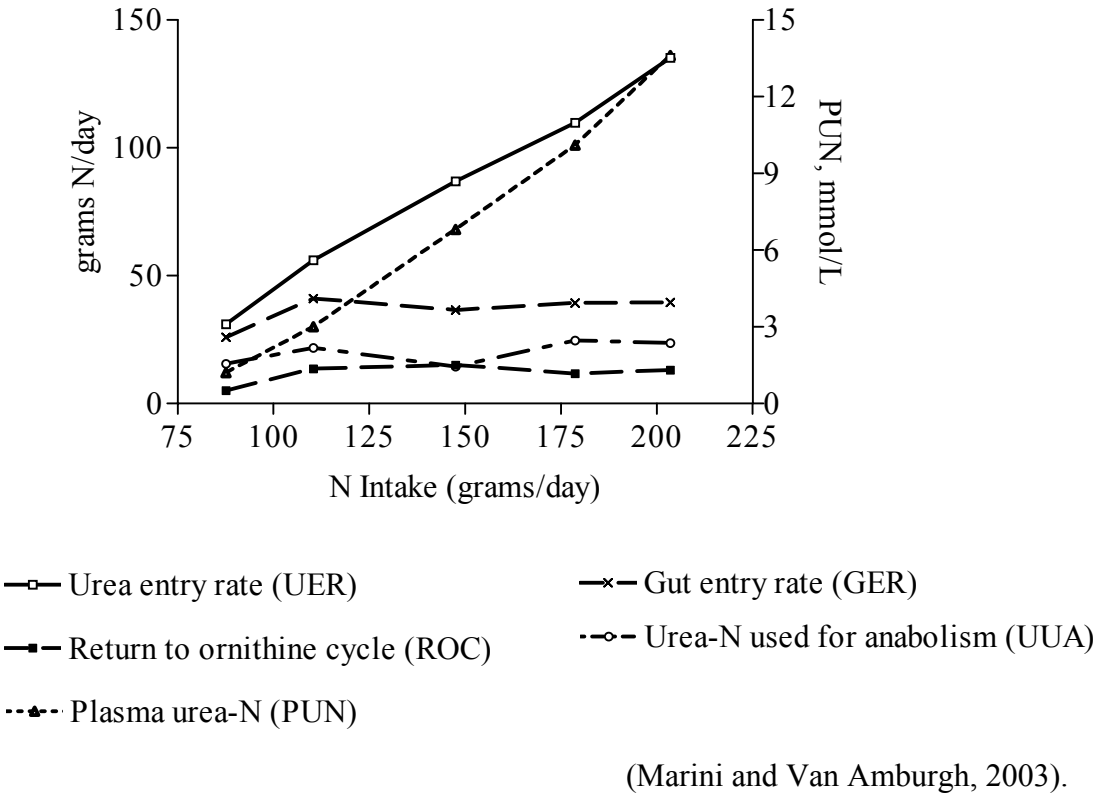
1.12 Factors Affecting Recycling of Urea into GIT

1.12.1 Dietary Protein Content and Intake

Studies by Kennedy and Milligan (1980) suggest an inverse relationship between dietary protein intake and urea-N entry into the rumen. Thus at low protein intakes and when fed a low quality roughage diet, the animals tend to have decreased blood concentrations of urea-N, decreased hepatic urea synthesis, decreased urinary urea nitrogen excretion and decreased transfer of urea to post stomach tissues (Bunting et al, 1987; Huntington, 1989; Marini and Van Amburgh, 2003). Archibeque *et al*., 2001 evaluated the effects of two forage species, each having two N levels on urea kinetics

and N metabolism and concluded that efficiency of N use is greater at low N intakes even though the absolute movements of N through the system increased with N intake.

In sheep, high levels of feed intake and with improved diet quality (hay-grass pellets/concentrate-forage), the proportion of urea-N entering the GIT (60-70%) and the proportion used for anabolic purposes (45-50%) is not affected. However the absolute amount of urea entering the GIT (recycling) and the amount returned to ornithine cycle increased with intake and for the concentrate-forage diet (Sarraseca *et al*., 1998; Lobley *et al*., 2000). Whereas ruminants fed low quality hay or are kept on a low protein diet, urea recycling to the GIT is 80-90% of urea entry rate and a higher proportion of absorbed N is retained. (Bunting et al, 1987; Marini and Van Amburgh, 2003).



(Marini and Van Amburgh, 2003).

Figure 3. Urea recycling in Holstein heifers. Urea-N recycled to the GIT (GER), the portion of GER returned to the ornithine cycle (ROC) and urea-N used for anabolism (UUA) reached a plateau at around 110 grams of N/day. Above this level most of the urea-N is excreted in the urine.

1.12.2 Energy Content and Fermentability of the Diet

Increasing the fermentable carbohydrate fraction of the diet increases urea recycling to the rumen (Kennedy, 1980; Kennedy and Milligan, 1980; Huntington, 1989) and decreases urea transfer to post gastric tissues (Reynolds and Huntington, 1988). Thus supplemental grain, starch, dried pulp and sucrose as energy sources significantly increase urea degradation in the GIT, particularly in the rumen. This response may be due to a combination of factors such as a reduction in rumen ammonia concentration, an increase in the quantity and rate of the fermentation of the dietary organic matter in the rumen (Kennedy and Milligan, 1980). Intraruminal infusion of sucrose was found to increase propionate production in addition to lowering the rumen ammonia and plasma urea concentration and increasing urea recycling to the GIT. This propionate was in turn available for glucose production thus sparing amino acids for tissue growth (Obara and Dellow, 1993; Seal and Parker, 1996). Propionate infusion into abomasums seems to improve the N balance and increase urea entry to gut (Kim *et al*., 1999). Further studies will be required to determine if it was simply a rumen energy response or propionate specific.

1.12.3 Ammonia Concentration in the Rumen

Rumen ammonia concentration may have a direct effect (decrease permeability of ruminal epithelia) on urea entry to GIT or it may affect concentrations of other fermentation products that impede urea entry to the GIT. Some authors have suggested that high rumen ammonia concentrations may depress urease activity, even though no direct evidence currently exists. (Egan *et al*., 1984). The same authors hypothesized that a 'boundary layer effect' of ammonia and carbon dioxide at the rumen liquid: epithelial

interface may inhibit urea entry to rumen. This suggestion was based on observations that with high urease activity at the rumen epithelial interface, high levels of ammonia are generated at the rumen epithelial surface. The movement of ammonia into the rumen biomass or into the blood will therefore depend upon the pH of these two locations and the ammonia gradient between the extra cellular fluid of the rumen wall and the rumen liquid. The ratio of ammonia to ammonium ions and carbon dioxide to bicarbonate ions also may be potential determinants of entry of urea to rumen.

1.12.4 Plasma Urea Concentration and Urea Transfer

Another factor that may promote entry of urea into the GIT is plasma urea concentration. However, contradictory evidence exists in the literature regarding the relationship between plasma urea concentration and gut entry of urea. Earlier studies suggested a positive linear relationship between increases in urea entry to the GIT and plasma urea concentration. In a comprehensive analysis across studies, Lapierre and Lobley, (2001) showed that this relationship occur only at plasma urea concentrations of < 6 mM for sheep and < 4 mM for cattle, and above these concentrations, urea entry is inhibited presumably by the boundary layer effects of rumen ammonia. They also pointed out that the correlation between plasma urea concentration and gut entry of urea across a range of studies where plasma urea exceeded 6 mM is very low ($r^2 < 0.3$). A more direct assessment by Lobley *et al*., (1998), in sheep where the plasma urea level was elevated by acute infusion (4-5 hrs) of amino acids into the mesenteric vein, found the correlation of gut entry of urea with plasma urea to be weak ($r^2 = 0.21$). However, because their measurements were made over the short term (8.5 hours) infusion of amino acids and the protein content of the diet was high (14%), the resulting small

incremental changes may have been difficult to detect accurately. Thus, plasma urea concentration may have a more significant effect on recycling when the needs of the animal to conserve N are much greater. Here, a sigmoidal relationship may result.

1.12.5 Feed Processing

Theurer *et al*. (2002) observed an increase in urea-N recycling to the portal drained viscera (PDV/gut tissues) with a resultant decrease in urinary urea-N output in growing beef steers fed steam-flaked sorghum compared to dry-rolled sorghum. They also observed a greater proportion of PDV urea-N transferred to the rumen (77%) than to the small intestine (23%). In a companion study by this group, (Alio et al., 2000) employing similar feed processing techniques, total splanchnic output of urea-N decreased when the diet was steam flaked. This may be due to the fact that steam-flaking increases starch and CP digestibilities. Processing of feed may thus be synchronizing the starch and nitrogen supply to the rumen and reducing the absorption of ammonia thus increasing N retention (Huntington, 1997). Decreasing the flake density also increases ruminally fermented starch thus increasing the amount of urea recycled back to the rumen.

1.12.6 Hormonal Regulation

Insulin is the primary hormonal regulator of metabolism in animals, promoting net amino acid uptake and protein synthesis by various tissues at physiological concentrations (0.63 to 0.83ng/ml). At these concentrations, hepatic removal of pyruvate, alanine and glutamine is also reduced (Brockman and Laarveld, 1986).

Reynolds (1992) observed a decrease in liver urea production, liver removal of NH_3, liver removal of amino acid nitrogen and an increase in liver release of glutamate by beef steers given daily injections of growth-hormone-releasing factor (GRF). As a result of this physiological and biochemical alterations, N retention doubled. Urea flux across portal drained viscera also increased as a result of growth hormone treatment. These data suggest a potential role for growth hormone in the regulation of urea metabolism.

1.12.7 Physiological State of the Animal

Increased metabolic demands of the animal (pregnancy, lactation etc) also appear to increase the amount of urea recycled (by saliva and rumen wall) to the gut (Benlamlih and de Pomyers, 1989). This may be achieved by an increased capacity of urea transfer across the digestive mucosa. Ritzhaupt *et al*. (1997, 1998) reported the presence of bidirectional urea transport mechanisms in ovine colon and rumen epithelia, which may play an important role in transfer of urea into gut. All these observations suggest that the animal body itself is a main regulator of urea recycling.

1.12.8 Role of the Kidneys

The role of kidney in salvage of urea from excretion can be very important as demonstrated by observations that the feeding of low protein diets to heifers reduced urea clearance by the kidneys (Marini and Van Amburgh, 2003). The calculated values correspond to 47% of urea reabsorbed by the kidneys at low levels of N intake (1.45% N diet) compared to only 8% urea reabsorption for high (3.4%) amounts of N intake. This increase in urea reabsorption is consistent with observations in rats fed low N diets where expression of urea transporters in the inner medullary-collecting duct was also

enhanced (Isozaki *et al*., 1994). Urea reabsorption in the kidney tubules (through urea transporters) and regulation of osmolarity of urine (for water conservation) may be coordinated through various hormones (e.g. vasopressin or antiduretic hormone; Goodman, 2002).

1.12.9 Urea Transporters

The existence of specific urea transporters in ruminant tissues has only recently been investigated. Urea transport especially across GIT and kidneys appears to be tightly regulated. The identification of urea transport systems and urea specific signalling pathways across these tissue beds in a variety of species rats, humans and ruminants (Ritzhaupt *et al*., 1997, 1998; Bagnasco, 2000; Goodman, 2002; Marini and Van Amburgh, 2003) supports this hypothesis. Mammalian urea transporters have now been identified in erythrocytes (UT-B), the renal medulla (UT-A), brain, kidney, testis, urinary bladder, GIT tissues (rumen, colon) etc.

Most of the literature regarding urea transport mechanisms deals with urea as an essential solute crucial for production of concentrated urine. These studies suggest a vasopressin (antidiuretic hormone) induced regulation of urea transporters and water channels, which work together to conserve water (Goodman, 2002). Vasopressin increases urea reabsorption from the inner medullary collecting duct via activation of adenylate cyclase and the production of cAMP (Shayakul and Hediger, 2004).

Evidence of urea transport as a regulatory mechanism for N conservation or disposal in the GIT and liver is beginning to emerge. Ritzhaupt *et al*. (1997, 1998) reported the existence of a bidirectional facilitative urea transport system in ovine colon and rumen epithelia. Marini and Van Amburgh, (2003) also reported the presence of

urea transporters (UT-B) in the rumen wall of heifers, which was more when heifers were fed high N diets. They suggested that this occurred to balance the opposing changes in urease activity. Thus the high-N diet depressed urease activity but increased urea transporter expression to overcome the low rate of urea hydrolysis. But in a follow up study with lambs however, Marini *et al*., (2004) failed to detect a significant increase in urea transporter abundance by various tissues in response to increased N intake. Thus the process and factors controlling rumen urea transport activity still require further investigation.

1.12.10 The Role of Blood Flow

As is the case for many tissues, the rate of substrate delivery has a large influence on substrate removal, and in this connection, blood flow and dynamics could be influential on urea transfer to the rumen. Hinderer and Englehardt (1976) found an increased rumen influx of urea when carbon dioxide was bubbled through the digesta or butyric acid was infused into a test solution placed in the rumen. The permeability of the capillaries may not favor an effect of urea entry into rumen, but the effect of blood flow may also be due to the fact that blood passes through a large number of capillaries.

1.13 Techniques for Measuring Urea Recycling

The quantitative significance of endogenous urea recycling to the GIT has been an accepted fact for some time. Various in vitro and in vivo methods (eg saline filled rumen pouches, urea loading etc) have been used to investigate urea recycling. The most common methods, however has been techniques involving measurement of net arterio-venous differences and the use of isotopic tracers. This involves labeling the blood urea

pool by intravenous infusion of [^{14}C] or [^{15}N] urea. Urea transfer to the rumen was then determined from the amount of the [^{14}C] bicarbonate or [^{15}N] ammonia appearing in the rumen fluid (Leng and Nolan, 1984). The basic assumption is that the [^{14}C] bicarbonate or [^{15}N] ammonia produced will get evenly distributed in the rumen ammonia pool. Estimates of urea entry can also be confounded by the fact that the bacteria present at the rumen epithelium hydrolyse most of the labeled urea thus preventing uniform mixing of labeled urea with intracellular urea pools. This will result in an underestimate of the urea entry estimated by such methods.

Recently a new urea kinetic approach (Figure 4) has been developed (Lobley *et al.*, 2000; Sarraseca *et al*., 1998) as an extension of a technique previously used in humans (Jackson *et al*., 1984, 1993). This approach involves injection of [^{15}N^{15}N] urea and isotopomer analysis of urinary or blood [^{15}N^{15}N] (M+2), [^{14}N^{15}N] (M+1) and [^{14}N^{14}N] (M) urea. The earlier model used for humans overestimated [^{14}N^{15}N] urea transfers because it failed to account for multiple reentries of [^{14}N^{15}N] urea into the gut. Sarraseca *et al*., (1998) introduced a correction for multiple reentries of [^{14}N^{15}N] urea into the gut. Labeled urea entry to the gut a maximum of six occasions accounted for 99% of the label movements. This model can be used both for continuous or single dose protocols.

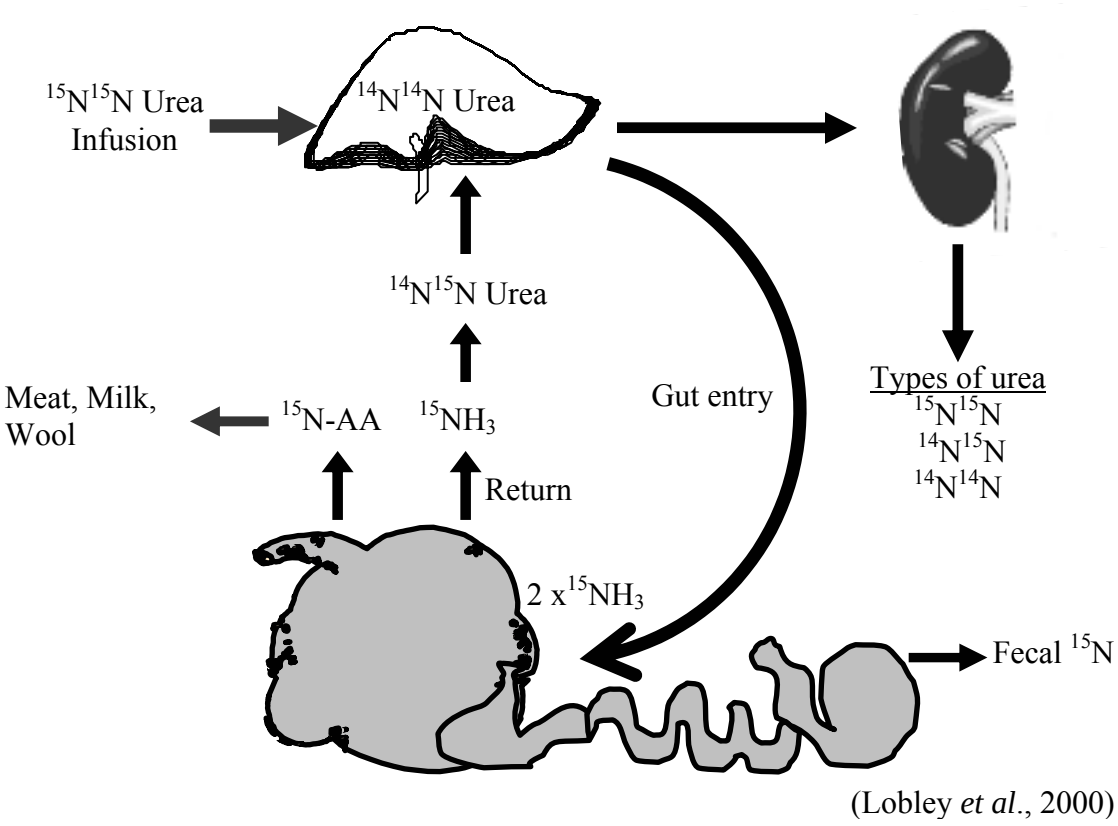
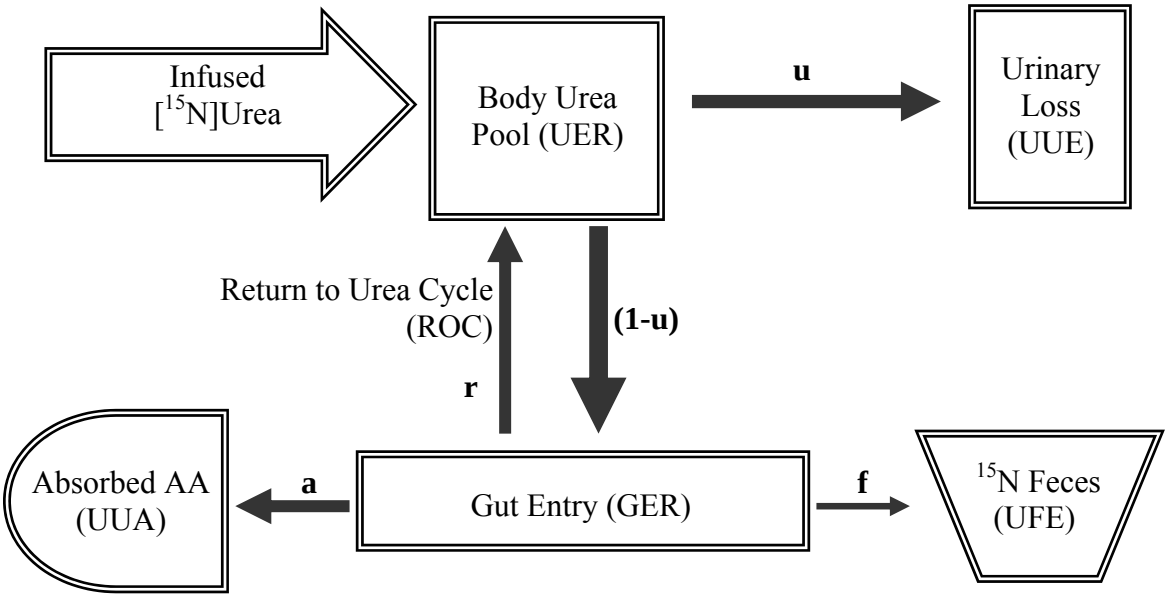


Figure 4. Urea kinetics based on infusion of [$^{15}N_2$]urea. Of the [$^{15}N^{15}N$] urea entering the body pool, a portion is transferred to the GIT where it is broken down by urease to two $^{15}NH_3$. These labeled ammonia molecules can either return to liver and combine with an unlabeled ammonia ($^{14}NH_3$) to form a [$^{14}N^{15}N$] (singly labeled) urea molecule or it can be excreted in the feces or it can be incorporated into amino acids and absorbed. The probability of an $^{15}NH_3$ combining with another $^{15}NH_3$ is considered to be negligible. The ratio of [$^{14}N^{15}N$]: [$^{15}N^{15}N$] urea in urine thus provides an estimate of the proportion of the urea flux broken down to ammonia and directly returned to the ornithine cycle.



(Lobley *et al*., 2000)

Figure 5. [$^{15}N_2$] Urea Kinetic Model. This figure depicts the two compartmental model based on the flow of [$^{15}N^{15}N$] urea. According to the model, vascular infused [$^{15}N^{15}N$] urea entering the GIT is converted to $^{15}NH_3$ by urease. The resulting $^{15}NH_3$ can either be used for the synthesis of [^{15}N] amino acids by microbes or it can be reabsorbed and converted to [$^{14}N^{15}N$] (M+1) urea in the liver. Isotopic dilution of [$^{15}N^{15}N$] urea in blood yields urea synthesis (urea-N entry rate; UER) by the liver, with the difference between UER and urinary urea elimination (UUE) the amount of urea transferred to the gut (gut entry rate; GER). The fractional transfers of UER to UUE and GER are represented by u and 1-u respectively. A portion of GER may return to ornithine cycle (ROC), while another portion may be excreted in feces (UFE). This difference between GER and these catabolic fates gives the urea-N used for anabolism (UUA; Lobely *et al*., 2000). In other words, ROC, UFE and UUA are each fractions of GER, which can be calculated by multiplying their respective fractional transfers (r, f and a) by the GER.

Rate of change of [$^{15}N^{15}N$]urea in the body

= rate of [$^{15}N^{15}N$]urea dose – loss rate in urine – rate of GIT transfer

$dh_{30}/dt = D_{30} – uh_{30} – (1 - u)h_{30}$

$= D_{30} – h_{30} = 0$

Rate of change of $^{14}N^{15}N$ urea in the body

= rate of [$^{14}N^{15}N$]urea dose – loss rate in urine – rate of GIT transfer

+ [$^{14}N^{15}N$]urea recycling + [$^{15}N^{15}N$]urea recycling

$dh_{29}/dt = D_{29} – u2h_{29} – (1 – u2)h_{29} + r2(1 – u2)h_{29} + r(1- u)h_{30}$

$= D_{29} – (1 – r2 + u2r2)\ h29 + r\ (1-u)\ h30 = 0$

D_{29}, D_{30}, h_{29} and h_{30} represent the quantities of [$^{14}N^{15}N$]urea and [$^{15}N^{15}N$]urea in the dose and the body respectively.

Information based on veno-arterial (VA) differences can be coupled with the above model for determining the partition urea-N to urine and to different compartments of gut. Gut metabolism can be divided by careful anatomical placement of catheters to isolate the small intestine (mesenteric drained viscera; MDV; Huntington, 1989; MacRae et al., 1997b; Seal and Parker, 1996) from the total GIT (Portal drained viscera; PDV). These measurements require major surgical interventions and also measurement of blood flow. But this approach can be used for both steady state and non-steady state conditions and also for trans-organ flux measurements of more than one metabolite. The information from the kinetic approach and the AV difference measurements can be coupled to obtain an indirect measure of urea transfer via saliva as the difference between GER and PDV net urea movements (Huntington, 1989).

Chapter 2: Materials and Methods

All the procedures used for the surgery and the conduct of the experiment were approved by the Animal Care and Use Committee, University of Maryland, College Park.

2.1 Animals and Diets

The experiment was conducted on growing wether sheep (20-25 kg BW), housed individually in rubber matted floor pens (1.5m x 2.5 m) bedded with wood shavings. The wood shavings were replaced every 2 weeks or more frequently if needed. Fresh water was made available ad libitum. Animals were kept on a pelleted ration (10 MJ metabolisable energy/kg dry matter, 145 grams crude protein/kg) fed to at least 2 times energy maintenance (0.4 MJ/ kg body weight $^{0.75}$) until about two weeks before the start of the experiment. Ten days prior to the trial, animals were gradually adapted to the pelleted experimental diet (Table 2) which they continued to receive through out the course of the study (2 months).

The experimental diet was low in protein (7.6% CP; on DM basis) but more or less adequate in fermentable energy (7MJ metabolizable energy/kg). Thus at 1.5 times energy maintenance intake (approximately 80 g feed/kg body weight$^{0.75}$), the diet provided 65% of metabolizable protein requirement of the animal. The protein content of the experimental diet was kept low to simulate a N limiting environment within the rumen and also to limit absorbed protein availability for microbial growth and protein deposition.

Under these conditions, it was expected that urea recycling would be greatest and metabolic pathways for urea recycling would be activated. Body weight and condition score of sheep were monitored frequently (at least once a week) to ensure that body weight was at least maintained on the low protein diet.

The experimental diet was fed (1000g/day) every two hours in equal proportions by automated feeder. Frequent feeding maintains steady-state rates of absorption and metabolism thus reducing fluctuations in blood nutrient concentrations and organ blood flows that normally occur with twice daily feeding. Under these conditions it is assumed that measurements made over 8 h (see below) will be more representative (i.e. constant) of the average daily metabolism. The reduced fluctuations in these parameters also reduces the measurement error (i.e. fewer animals are required).

Table 2. Composition of experimental diet fed to sheep [1]

Ingredient	% as fed
Corn Dent Yel grain	28.5
Cottonseed hulls	13.5
Beet pulp, dried	20.0
Wheat straw	36.5
Mineral mix	1.5
Laboratory analyses	
Dry matter, % of diet	90.6
Crude protein, % of DM	7.6
Starch, % of DM	23.2

[1]Contained (DM basis) 0.83% Ca, 0.3% P, 0.23% Mg, 366 PPM Fe, 146 PPM Zn and 85 PPM Mn. Each kg dry matter also contained 11,037 IU of vitamin A, 2200 IU of vitamin D and 36 mg of vitamin E

2.2 Surgery

Under general anesthesia, sheep were fitted with catheters in the distal and proximal mesenteric vein, hepatic portal vein and a femoral artery. After surgery, sheep were placed in individual floor pens for recovery to full intake and incision healing. Care of wounds and checks for catheter patency were performed daily. The arterial catheters were used throughout the experiment for collection of plasma samples for analysis of plasma urea enrichment and concentration, and also amino acid concentration.

2.3 Design and Protocol

Sheep were randomly assigned to four treatments (Control and three levels of urea) in a balanced 4×4 Latin square design with 10-day treatment infusion periods. Four days separated each treatment period, during which animals were housed in floor pens to avoid treatment interactions and to provide exercise. The control treatment involved a constant infusion (350 ml/d) of saline into the jugular vein and the urea treatments (8, 16 and 24 g urea/d or 3.76, 7.52, and 11.28 g urea-N/d respectively) involved constant infusions (urea in 350 ml/d) into the jugular vein. The different levels of urea were selected on an incremental basis to make up for the deficiency in metabolisable protein supply on the low protein basal diet. Thus, at the highest level of urea infusion, animals were projected to receive ~ 115 g MP/day which is 25% above predicted requirements (92 g MP/day) for growing sheep gaining 250g/day. Here we assumed each unit of urea-N infused was equivalent to a unit of metabolisable protein N.

2.4 Isotope Infusions and Analysis

Two days prior to the start of a treatment period (10 days), animals were transferred to metabolism crates where they were maintained for the next ten days. A temporary catheter was inserted into a jugular vein 1 to 2 days prior to infusion of control or treatment (levels of urea) solutions. All the solutions for intra-jugular infusion were prepared in double-distilled water and the pH adjusted to 7.4. These solutions were then filtered through a 0.45 μm syringe filter (Nalgene, Rochester, NY) into a sterile glass bottle. Further more, solutions were infused intravenously through an inline 0.2 μm syringe filter (Nalgene, Rochester, NY) and filters replaced every 2-3 days. Patency of temporary catheters was also checked daily. From day 8 to 10 of each treatment period (ie. the last 80 hrs of each treatment period), [$^{15}N^{15}N$] urea (99 atoms % ^{15}N; Cambridge Isotopes Laboratories, Inc. MA, USA) was infused along with the unlabeled urea (Table 3) to trace the metabolic fates of urea through the various body pools. The amount of [$^{15}N^{15}N$] urea infused with each treatment was adjusted in order to increase [$^{15}N^{15}N$] urea enrichments (M+2) in urine to approximately 3 atom percent excess (APE) at 'plateau' (steady state).

Table 3. Amount of unlabeled and labeled urea infused into the animal during a ten day treatment period

Treatment (g urea/d)	day 1 to day 6 Unlabeled urea (g/d)	day 1 to day 6 [1]Labeled Urea (g/d)	day 7 to day 10 Unlabeled Urea (g/d)	day 1 to day 6 [1]Labeled Urea (g/d)
Control	0.34	0	0	0.34
8	8	0	7.47	0.53
16	16	0	15.21	0.79
24	24	0	22.98	1.02

[1]Quantity of [$^{15}N^{15}N$] urea calculated to attain an enrichment of 3 atom percent excess

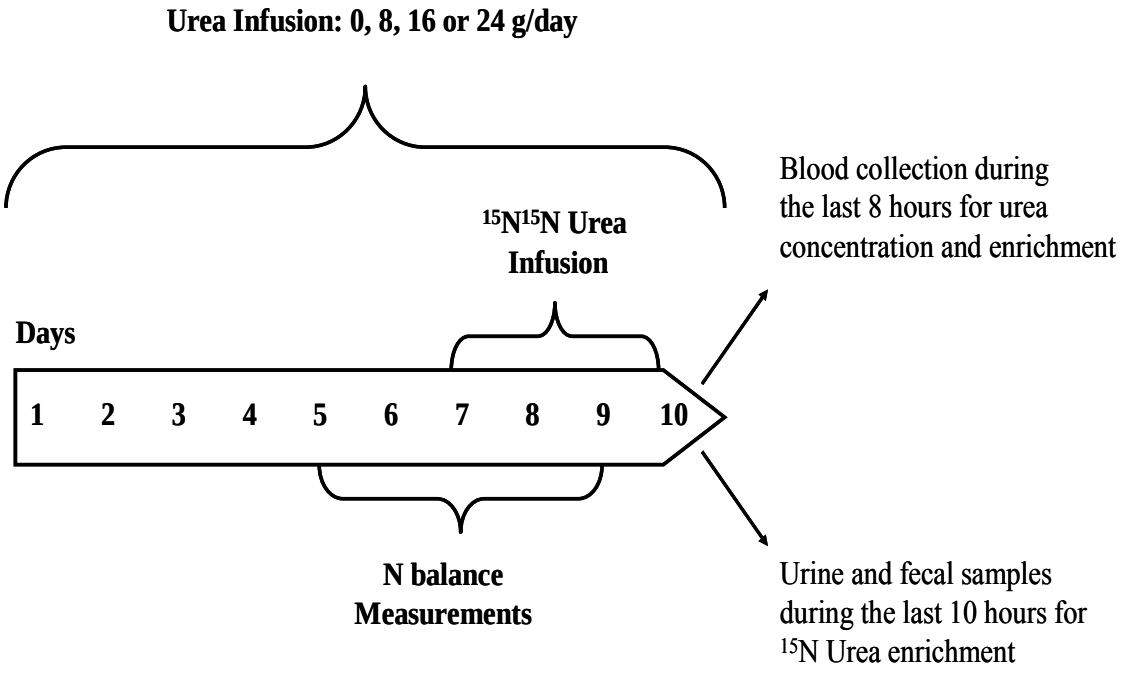


Figure 6. Tracer Infusion and sampling protocol during treatment periods. Unlabeled urea was infused throughout the 10 days and a portion replaced with labeled urea over the last 80 hrs. N balance measurements were taken on day 6, 7, 8 and 9. Blood and plasma samples for concentration and enrichment, and fecal samples for enrichment, were collected on day 10 during the last 10 h of infusion.

On day 5 of each period, sheep were fitted with a light harness for total collection of urine by suction and feces by bag**.** Total urine and feces were collected on days 6, 7, 8 and 9. Urine was aspirated by vacuum pump into plastic bottles containing 100 ml of 4M HCl to prevent volatilization of N from urine as ammonia. Urine containers and fecal bags were emptied daily, mixed thoroughly, and sub-samples (5% and 20% of total for urine and feces respectively) taken and stored at -20^{o}C until analyzed for total N and total ^{15}N enrichment. A sub sample of the daily urine output was also taken for determination of urine urea concentration (100 µL of urine + 100 µL of [$^{13}C^{15}N_2$] urea standard; accurately weighed) and enrichment (1ml acidified urine). Briefly, to a known weight (0.1g) of urine was added an equal known weight of a solution containing [$^{13}C^{15}N_2$] urea (5 mg/ml) and sample mixed and stored frozen at -4^{o}C.

Prior to the start of [$^{15}N^{15}N$] urea infusion (day 7) plasma, urine and fecal samples were collected for measurement of ^{15}N natural abundances. Blood samples (4 ml) were collected, every hour over the last 8 hours of isotope infusion (eight samples). Blood was centrifuged at 4000 rpm for 15 minutes at 4^{o}C to separate plasma for determination of urea and amino acid concentration, and urea enrichments. For amino acid and urea concentrations to a known weight (0.5g) of plasma was added a mixture containing U-^{13}C amino acids (from hydrolysis of algae; Table 5) of amino acid and urea standard (Table 4). Urea samples were also collected every 2 hours for determination of urinary urea enrichments (5 samples).

Table 4. Composition of amino acid and urea standard for measurement of concentration in plasma[a]

0.75 mg hydrolyzed [U-^{13}C] algae [b]

100 nmol L-tryptophan-[indole-D_5] [c]

200 nmol L-glutamine-amide-^{15}N [c]

25 nmol S-methyl-D_3- methionine [c]

7 μmol [^{13}C-$^{15}N_2$]-urea [c]

[a]Composition is for each 0.5 g of plasma

[b]Algae has all the amino acid carbons universally labeled with ^{13}C (99 atoms %; Martek Biosciences Corp., Colombia, MD)

[c]From Cambridge Isotope Laboratories, Inc. MA, USA

Table 5. Amino acid composition of hydrolyzed [U-^{13}C] algal solution

Amino Acid	μmoles/g algal solution
Alanine	0.297
Arginine	0.129
Aspartic acid	0.247
Glutamic acid	0.266
Glycine	0.307
Histidine	0.057
Isoleucine	0.085
Leucine	0.232
Lysine	0.310
Methionine	0.037
Phenylalanine	0.101
Proline	0.174
Serine	0.190
Threonine	0.156
Tyrosine	0.083
Valine	0.158

Feces was collected (single sample) over the last 10 hours of isotope infusion, thoroughly mixed and a sub sample (100g) taken for estimation of total ^{15}N enrichment. All samples were kept frozen until analyzed.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Urine and Plasma Samples for Urea Enrichment and Concentration

Urine and plasma samples were acidified by adding equal volume of 10 % TCA and 15% sulpho salicylic acid (w/v), respectively, and centrifuged for 10 min at 10,000 rpm to precipitate proteins and other debris. The supernatant was desalted by ion-exchange by application to 0.5 g of cation exchange resin (AG-50, 100-200 mesh, ×8, H^+ form; Biorad, Richmond, CA, USA). The resin was washed with 2 x 3 ml of water and urea and amino acids eluted with 2 ml of ammonium hydroxide plus 1 ml of double distilled water. An aliquot (50 µl) of the elute was blown down under N_2 gas and tertiary-butyldimethylsilyl derivatives of urea was prepared by adding 50 µl each of acetonitrile (Pierce chemicals) and N-methyl-N-t-butyl-dimethylsilyl-trifluroacetamide (Pierce chemicals) and then heating at 90^{o}C for 20 minutes. Electron impact ionization (EI mode) GC-MS (5973 mass selective detector coupled to a 6890 series GC system; Agilent; Palo Alto, CA) was used for determining $^{15}N^{15}N$ and $^{14}N^{15}N$ urea enrichment and concentration in these samples. Ions corresponding to mass-to-charge ratio (m/z) 231.2 (unlabeled; M), 232.2 (singly labeled; M+1), 233.2 (doubly labeled; M+2), and 234.2 (internal tracer standard; M+3) were monitored using selected ion monitoring (SIM). Samples were injected in the split mode (40:1) and separation effected on a capillary column (EC-1; Alltech, Deerfield, IL) with conditions of: an initial temperature of 150^{o}C followed by 15^{o}C/min to 250^{o}C. Total run time was 6.67 min. Urea

concentration in samples was calculated based on the ratios of M+3/ M after correcting for spill over of M+ 2 to M+3. A spill-over curve of M+2/M and M+3/M ratios was constructed using increasing [$^{15}N^{15}N$] urea enrichment standards, the slope and intercept of which were used to calculate spill-over of M+2 to M+3 (Figure 8).

2.6 Gas Chromatography-Mass Spectrometry Analysis of Plasma Samples for Amino Acid Concentration

After aliquots for determining plasma urea concentrations were taken, the remaining elutes were lyophilized overnight to concentrate the amino acids. To these lyophilized samples, 200 μL of 0.1N hydrochloric acid was added, which was then transferred to a V-vial and blown down using N_2 gas (2 psi) at 40^{o}C. Tertiary-butyldimethylsilyl derivatives of the sample were prepared by adding 80-120 μL (1:1 ratio; depending upon the sample) of dimethylformamide (Pierce chemicals) + N-methyl-N-t-butyl-dimethylsilyl-trifluroacetamide (Pierce chemicals) and then heating at 90^{o}C for 30 minutes. GC-MS (EI mode) was used for determining amino acid concentrations. One μL of the derivatized sample was used for injection in a split mode at 40:1 split. The capillary column (EC-1; Alltech, Deerfield, IL) was initially held at 100^{o}C and ramped up at the rate of 10^{o}C/minute to a final temperature of 300^{o}C with a total run time of 23 minutes. Selected ion monitoring (SIM) was used to determine the atom percent excess (APE) by monitoring ions (Table 6) of each amino acid.

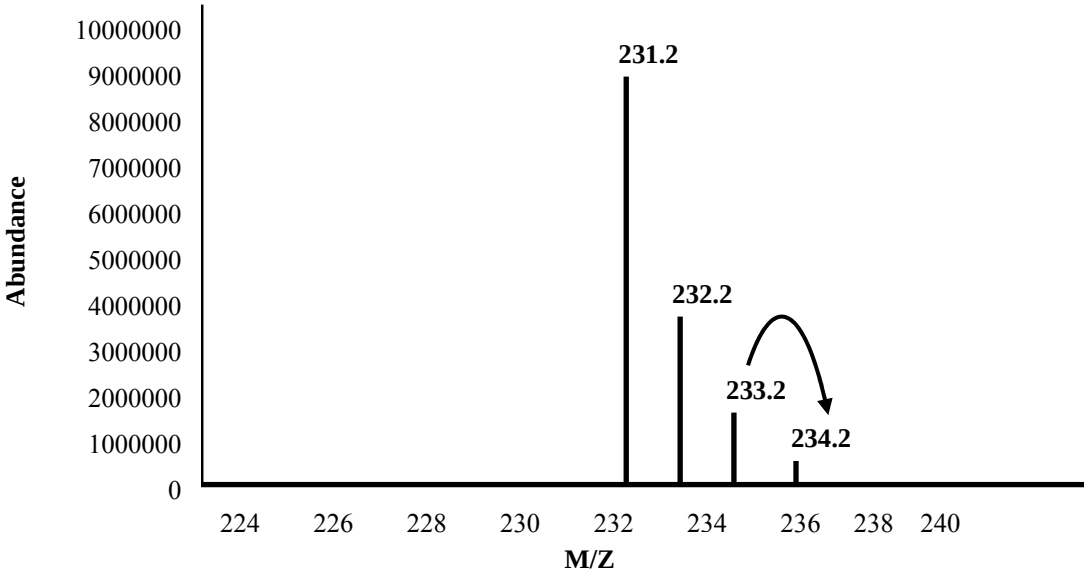


Figure 7. Ion spectra of a sample containing doubly labeled urea molecule. 231.2 (M) is the unlabeled ion, 232.2 (M+1) is a singly labeled ion and 233.2 (M+2) is a doubly labeled ion. The ratio of abundances of these ions gives the relative enrichments of M+1 and M+2 in a sample. A portion of M+2 spills over to M+3 which is corrected for using an M+2 spill over curve.

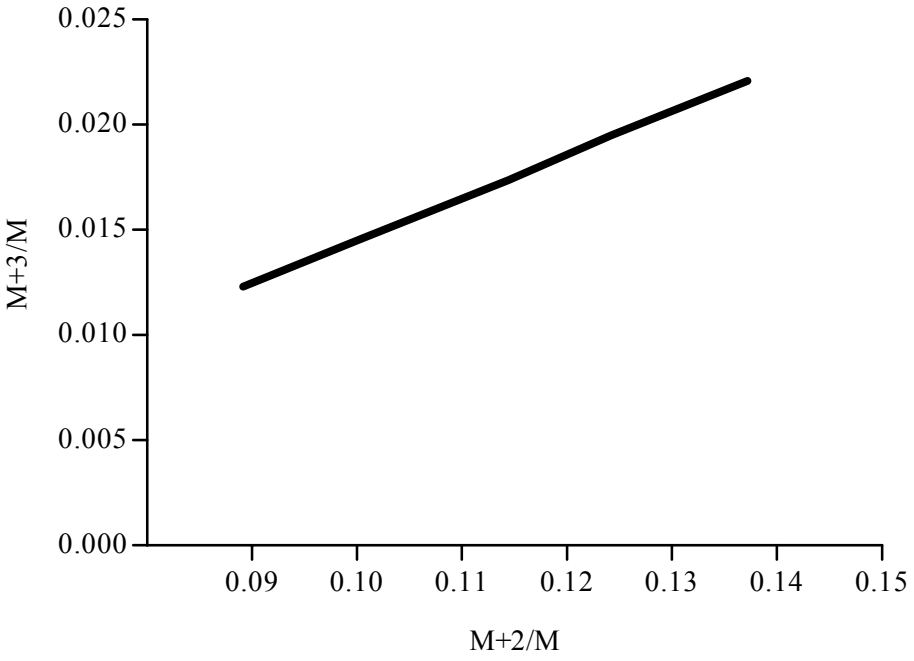


Figure 8. Curve for correcting spill-over of M+2 to M+3. The curve was constructed using increasing [$^{15}N^{15}N$] urea enrichment standards and the ratio M+2/M plotted against M+3/M. The spill over ratio is calculated as M+2/M × slope + intercept. This ratio is subtracted off from M+3/M ratio to obtain the corrected M+3/M ratio.

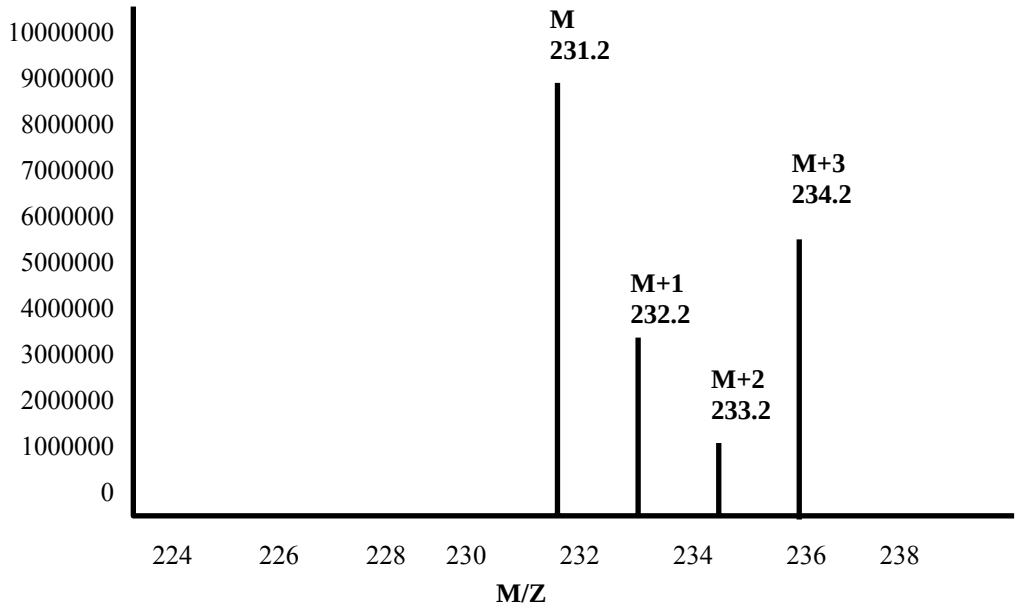


Figure 9. Ion spectra produced when there is [$^{13}C^{15}N_2$] urea in the sample as an internal standard. The internal standard appears at the M+3 ion 234.2, the ratio of which with M is taken and corrected using a series of concentration standards to determine the urea concentration in the sample.

Table 6. Ions monitored for individual amino acids in plasma

Amino acid	Ions Monitered[1]
L-Alanine	M = 260 & M+3 = 263
L-Aspartic Acid	M = 302 & M+2 = 304
L-Glutamic Acid	M = 432 & M+5 = 437
L-Glutamine	M = 168 & M+1 = 169
Glycine	M = 246 & M+2 = 248
L-Histidine	M = 440 & M+6 = 446
L-Isoleucine	M = 302 & M+6 = 308
L-Leucine	M = 302 & M+6 = 308
L-Lysine	M = 300 & M+6 = 306
L-Methionine	M = 292 & M+4 = 295
L-Phenylalanine	M = 234 & M+8 = 242
L-Proline	M = 286 & M+5 = 291
L-Serine	M = 390 & M+3 = 393
L-Threonine	M = 404 & M+4 = 408
L- Tryptophan	M = 244 & M+4 = 249
L-Tyrosine	M = 302 & M+2 = 304
L-Valine	M = 288 & M+5 = 293

[1]M, unlabeled amino acid; M+n, internal tracer standard

2.7 Analysis of Fecal Sample for ^{15}N Enrichment

Fecal samples were freeze-dried to desiccate out all the moisture and powdered before analyzing it on the mass spectrometer. ^{15}N analysis was performed using a CF-IRMS (continuous flow-isotope ratio mass spectrometer) by Rittenberg technique. In this method, alkaline hypobromite is used to oxidize NH_4^+-Nto N_2 in the absence of air.

2.8 Analysis of Total N in Urine and Feces

For chemical analysis, urine and fecal samples were freeze-dried and finely ground. N content in urine and feces were determined using an automated N analyzer (LECO CN-2000).

2.9 Calculations

All calculations for urea kinetics were adapted from Lobley et al., (2000). Assumptions of steady state and constant urea pool size during a period were followed for the calculations. Changes in pool size of urea were accounted for by replacing an equal amount of unlabeled urea for an equal amount of label during the last 80 hours of infusion.

The [$^{15}N^{15}N$] urea used for infusion was checked for isotopic purity. The purity of the tracer was measured to be 96.61 APE (M+2) using mass spectrometry. Urea fluxes were calculated using the following equations.

$$\text{Urea entry rate (UER)} = \left(\frac{E_{D30}}{E_{U30}} - 1\right) D_{30} \quad (1)$$

where E_{D30} and E_{U30} are the enrichments of [$^{15}N^{15}N$] urea in the dose and urine respectively and D_{30} is the rate of infusion of the dose (mass/time).

$$\text{Urinary urea elimination (UUE)} = \text{urea concentration in urine} \times \text{urine output} \quad (2)$$

$$\text{Fractional transfer of UER to urine (u)} = \frac{UUE}{UER} \quad (3)$$

$$\text{Gut entry rate (GER)} = UER - UUE \quad (4)$$

$$\text{Proportion of GERthat reenters ornithine cycle (r)} = \frac{\rho}{(1-u)} \quad (5)$$

$$\text{where } \rho = \frac{UUE_{29}}{(UUE_{29} + UUE_{30})}$$

where UUE_{29} is the amount of $^{14}N^{15}N$ excreted in urine

and UUE_{30} is the amount of $^{15}N^{15}N$ excreted in urine

$$\text{Return to ornithine cycle (ROC)} = r \times GER \text{ (or) } \rho \times UER \quad (6)$$

$$\text{Fraction of GER excreted in the feces (f)} = \frac{(u \times UFE)}{(1-u)(UUE_{29} + UUE_{30})} \quad (7)$$

where UFE is the amount of ^{15}N (mass/time) excreted in feces.

Urea-N utilized as absorbed amino acids (a) is calculated indirectly as a difference measurement.

$$a = 1 - r - f \quad (8)$$

Thus the product of fractional transfers and GER yeilds the absolute amounts partitioned towards the respective metabolic fates (anabolism, ROC, feces).

2.10 Statistical Analysis

Data were analyzed by ANOVA for a Latin square design. The following model was used:

$Y_{ijk} = \mu + T_i + C_j + R_k + \epsilon_{ijk}$ Where

Y_{ijk} = response variable (UER, GER, etc)

μ = grand mean

T_i = treatment effect

C_j = random effects (animal, period)

R_k = effect of period.

ϵ_{ijk} = residual error

Significance was tested at $P < 0.05$ ($\alpha = 5\%$).

Data were analyzed using the PROC MIXED procedure of SAS (SAS Ins., Inc., Cary, NC) with animal and period blocking factors. For the analysis, treatment was taken as a fixed effect, and animal and period as random effects. Least squares means were obtained for each treatment and linear or quadratic effects of response variables with each level of urea infusion tested by constructing orthogonal polynomial contrasts. Pair-wise mean comparisons were done using the Tukey-Kramer adjustment.

Chapter 3: Results

3.1 Nitrogen Balance

There were no feed refusals by any sheep on any of the treatments even though the experimental diet was low in crude protein. N digestibilities ranged from 30 – 41% and increased ($P = 0.016$; Table 7) with each level of urea infusion. The low values for N digestibilities may be due to the type of experimental diet fed to the sheep or due to its affect on various rumen factors involved in nutrient utilization. Dry matter digestibilities also showed a similar response as N digestibilities, and increased with increasing levels of urea ($P = 0.002$). During Control period, sheep were in positive N balance (1.45 g N retained/d), and N retention increased (< 0.001) with each level of treatment reaching a maximum (5.18 g N retained/d) by the third level (7.52 g urea-N/d). With increasing levels of urea-N infused, the amount of N excreted in the urine increased ($P < 0.001$, Table 7). The absolute ($P < 0.001$) and proportional ($P < 0.001$) amounts of urea-N in urine also increased with each level of urea infusion, indicating that most (%) of the excess N infused into the animal was excreted as urea-N. Thus urea-N accounted for 80% of total N excreted at the highest level of urea infusion compared to 40% for the Control. Fecal N showed a tendency to nadir even though the statistical analysis showed a significant difference between treatments ($P = 0.016$) which corresponds well with the increasing N digestibilities with each treatment.

Table 7. N balance measurements in sheep (n = 4) kept on a low protein diet and infused with four levels of urea

	Treatment (g urea/day)[1]					
N balance measurements[2]	0	8	16	24	SED	*P*
N intake (g /d)	10.67	10.67	10.67	10.67		
N from urea infusion (g /d)	0.16	3.76	7.52	11.28		
N in feces (g /d)	7.48^{a}	6.61^{ab}	6.38^{b}	6.26^{b}	0.370	0.016
N in urine (g /d) (A)	2.21^{d}	4.12^{c}	6.63^{b}	10.23^{a}	0.327	<0.001
Urea-N in urine (g /d) (B)	0.94^{d}	2.48^{c}	4.95^{b}	7.79^{a}	0.256	<0.001
Urea-N in urine (%) (B/A*100)	40.4^{c}	59.6^{b}	73.2^{a}	80.2^{a}	3.43	<0.001
N retained (g /d)	1.45^{c}	3.69^{b}	5.18^{ab}	5.46^{a}	0.446	<0.001
N digestibility (%)	29.9^{b}	38.0^{ab}	40.2^{a}	41.3^{a}	3.47	0.016
DM digestibility (%)	47.5^{b}	55.0^{a}	56.0^{a}	58.9^{a}	2.73	0.002

[1]Treatment means with different superscripts are significantly different from each other
[2]N balance measured over a period of 4 days

3.2 Urea Enrichments

Enrichment of [$^{15}N^{15}N$] urea in urine samples collected on day 8, 9 and 10 of the experimental period were not significantly different from each other. Thus enrichments of urinary [$^{15}N^{15}N$] urea reached isotopic plateau by the second day of tracer infusion (~24-30 hours; Figure 10). One the contrary, the enrichments of [$^{14}N^{15}N$] urea continued to rise and appeared to plateau only by the last day of tracer infusion (72-80 hours from the start of tracer infusion; Figure 10). The plasma pool enrichments followed the same trend and were not significantly different from those in the urine for both [$^{15}N^{15}N$] and [$^{14}N^{15}N$] urea (Figure 11). However, the fecal ^{15}N enrichments were significantly different ($p < 0.001$) on all the four days of tracer infusion and thus did not attain isotopic steady state even after 80 hours of tracer infusion (Figure 12).

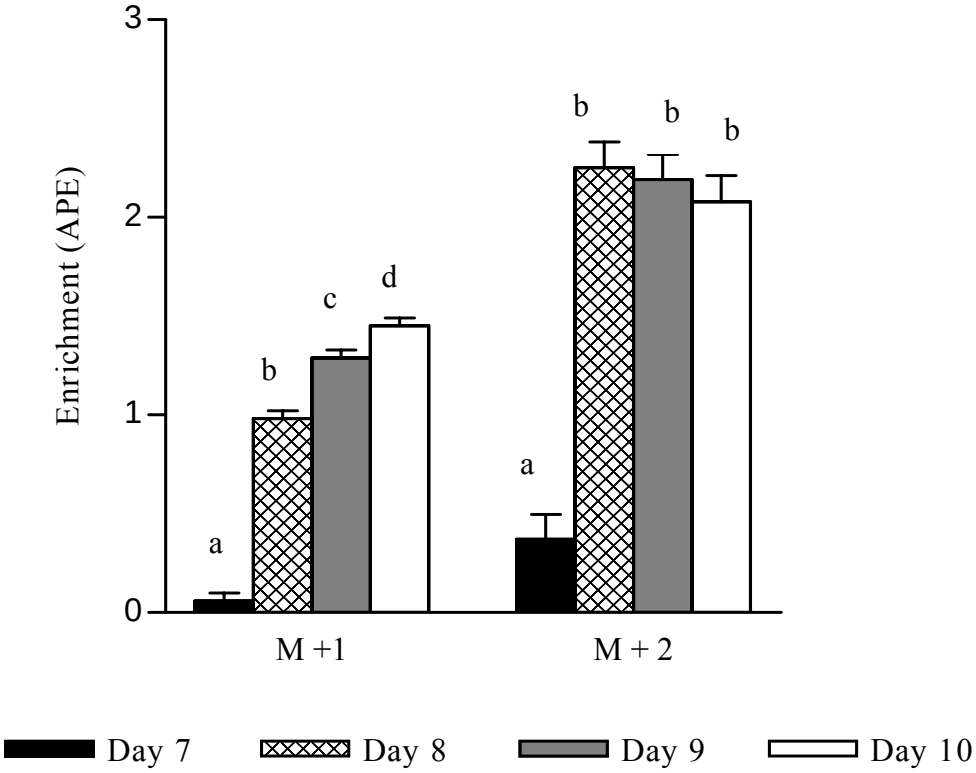


Figure 10. Overall means of singly (M+1) and doubly labelled (M+2) urea in urine over four days of tracer infusion. [$^{15}N^{15}N$] urea enrichments reached a plateau by the second day of infusion were as [$^{14}N^{15}N$] urea enrichments reached a plateau only by the last day of tracer infusion.
Each bar represents the mean of treatment values for that particular day of urea infusion.
Bars within each group (M+1 or M+2) having different superscripts are significantly different from each other.

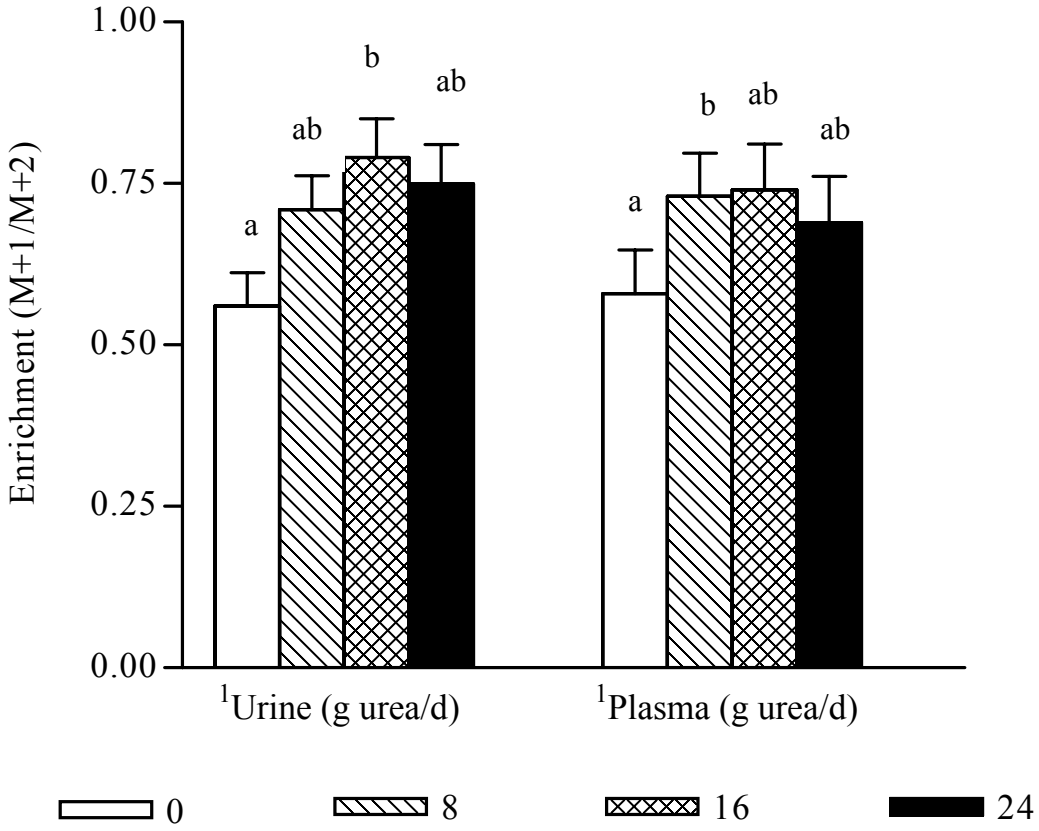


Figure 11. Treatment means for urine and plasma ratios of singly to doubly labeled urea [$^{14}N^{15}N$]:[$^{15}N^{15}N$].

[1]Mean enrichments of [$^{14}N^{15}N$] and [$^{15}N^{15}N$] urea for each level of infusion are not significantly different in urine and plasma. Vertical bars represent treatment means in either urine or plasma. Bars within each group (urine or plasma) with different alphabets are significantly different from each other.

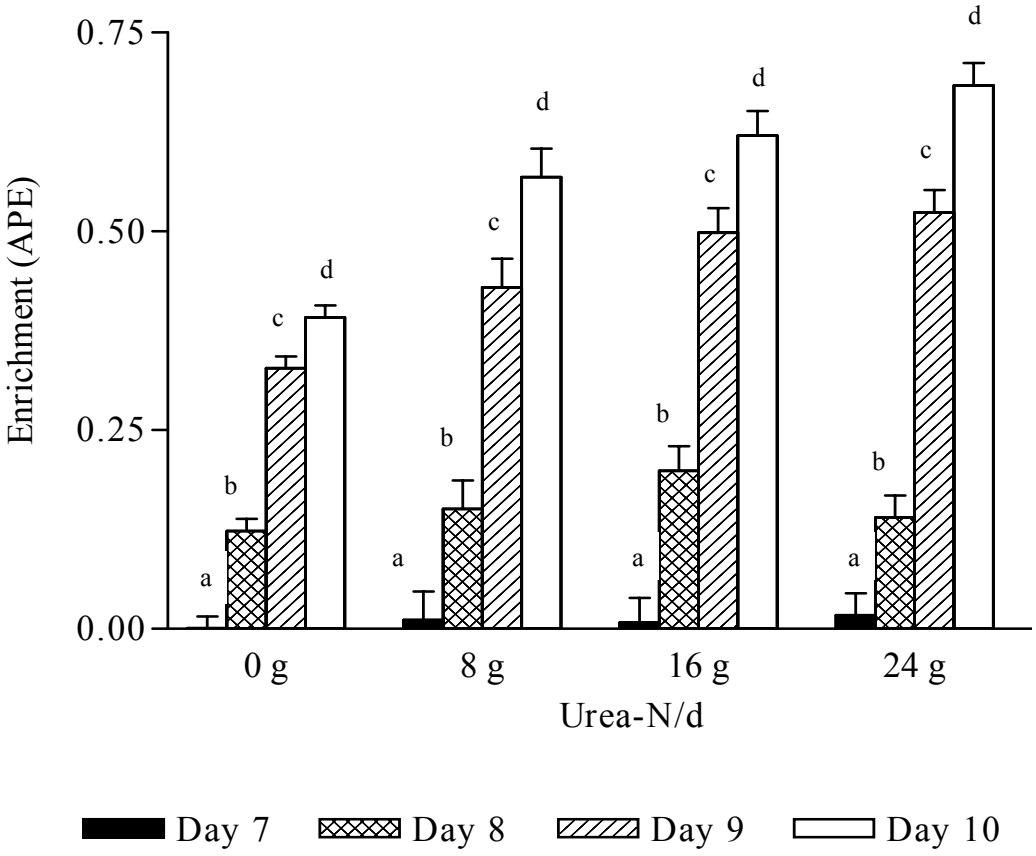


Figure 12. Treatment means for fecal total ^{15}N (0, 8, 16, 24 g urea/d) enrichments on days 7, 8, 9, 10 of [$^{15}N^{15}N$] urea infusion.

Fecal ^{15}N enrichments did not reach plateau even after eighty hours of tracer infusion for any of the four treatments.

Vertical bars represent treatment means. Bars with different superscripts have significantly different treatment means.

3.3 Urea Kinetics

As expected, urea entry rate (UER), the sum of hepatic ureagenesis and urea infusion increased ($P < 0.001$) with increasing urea infusion rates (Table 8). As UER increased, the amount (GER; $P < 0.001$) but not the proportion (1-u; $P = 0.014$) of urea-N partitioned to the GIT also increased. The portion of UER excreted in the urine increased both quantitatively (UUE; $P < 0.001$) and also on a fractional basis (u; $P = 0.014$). Thus fractional transfer of urea back to GIT was highest (81%) at the lowest level of urea infusion and went down to 63% at the highest level. However the amount of ammonia returning to ornithine cycle and contributing to urea resynthesis (ROC) after hydrolysis of urea in the GIT showed a significant increase with each level of urea infusion ($P < 0.001$). The fractional return of urea derived ammonia for hepatic ureagenesis also showed the same trend ($P <0.001$) indicating that the ability of rumen microbes to capture recycled-N had been reduced. The amount of urea excreted in the feces (UFE), even though statistically different across treatments ($P = 0.002$) attained a plateau by the third level of urea infusion as was suggested by the significant quadratic component for means ($P = 0.047$). The proportion of GER to UFE (0.001) was highest (21%) at the lowest level of infusion and this declined to 11 % at the highest level corresponding well with N balance measurements. Urea utilized for anabolism (UUA), i.e. urea absorbed as amino acids (from microbial protein synthesis) showed a tendency to increase with increasing levels of urea supply ($P = 0.003$).

However the values for UUA did not differ significantly between the last three levels of urea thus suggesting a limitation to microbial protein synthesis in the rumen. Also the fractional contribution of GER to UUA decreased with increasing urea supply (P = 0.003) and remained the same for the last two levels. Thus most of the urea recycled back to the GIT is directed towards catabolic fates.

3.4 Plasma Amino acid Concentrations

There is a general trend for concentrations of amino acids to increase with levels of urea infusion (Table 9). In particular, plasma concentrations of leucine (P = 0.014), lysine (P = 0.050), methionine (0.050), phenylalanine (0.008) and tryptophan (0.016) i.e. amino acids considered to be potentially limiting in microbial protein increased significantly with levels of urea infusion with most of then attaining a maximum value by the third level (16 g urea/d) of infusion. The trends in concentrations of limiting amino acids followed the increase in N balance. Surprisingly, histidine concentrations were not significantly affected by urea infusion. Of the non-essential amino acids, only for proline plasma concentration levels increased (P = 0.047) by urea infusion.

Table 8. Urea Kinetics in growing sheep given infusions of unlabelled urea into the jugular vein[1]

	Treatment (g urea/d)[2]					
	0	8	16	24	SED	*P*
Urea flux measurements (g urea-N/d)						
UER[3]	5.05[d]	10.56[c]	16.98[b]	21.78[a]	0.619	<0.001
UUE[4]	0.95[d]	2.54[c]	5.44[b]	8.11[a]	0.304	<0.001
GER[5]	4.11[d]	8.02[c]	11.28[b]	13.67[a]	0.507	<0.001
ROC[6]	1.82[d]	4.42[c]	7.40[b]	9.18[a]	0.405	<0.001
UFE[7]	0.86[c]	1.18[b]	1.48[ab]	1.52[a]	0.102	0.002
UUA[8]	1.43[b]	2.42[a]	2.50[a]	2.98[a]	0.320	0.003
Fractional transfers of urea-N						
UER to urine (u)	0.187[c]	0.244[bc]	0.327[ab]	0.372[a]	0.023	0.014
UER to GIT (1-u)	0.813[a]	0.756[ab]	0.673[bc]	0.628[c]	0.029	0.014
GER to ROC (r)	0.439[c]	0.550[b]	0.657[ab]	0.672[a]	0.029	<0.001
GER to UFE (f)	0.211[a]	0.152[b]	0.131[bc]	0.111[c]	0.159	<0.001
GER to UUA (a)	0.350[a]	0.298[ab]	0.218[bc]	0.218[c]	0.024	0.003

[1]Values are the mean of four observations
[2]Treatment means with different superscripts are significantly different from each other
[3]UER, urea entry rate
[4]UUE, urinary urea elimination
[5]GER, gut entry rate
[6]ROC, return to ornithine cycle
[7]UFE, urea-N in feces
[8]UUA, urea-N utilized for anabolism

Table 9. Plasma concentrations of amino acids and urea and urea clearance in sheep

	Treatment (g urea/d)					
	Control	8	16	24	SED	*P*[1]
Essential Amino acids (μM)[2]						
Histidine	112.7[a]	96.6[a]	108.9[a]	99.1[a]	10.16	NS
Isoleucine	86.9[b]	136.8[a]	153.0[a]	126.7[ab]	11.45	0.014
Leucine	163.1[a]	167.7[a]	199.1[a]	158.3[a]	14.16	NS
Lysine	128.2[b]	169.6[ab]	202.0[a]	169.8[ab]	19.79	0.050
Methionine	30.3[b]	39.9[ab]	47.5[a]	45.1[ab]	4.49	0.050
Phenylalanine	100.4[a]	92.1[ab]	96.0[a]	81.0[b]	4.56	0.008
Threonine	93.9[a]	105.9[a]	142.0[a]	125.5[a]	25.01	NS
Tryptophan	28.7[b]	43.4[ab]	52.3[a]	30.0[b]	5.71	0.016
Valine	251.8[a]	278.6[a]	324.0[a]	265.7[a]	25.99	NS
Non essential Amino acids (μM)[2]						
Alanine	291.7[a]	318.6[a]	326.9[a]	287.4[a]	25.50	NS
Aspartate	15.4[a]	15.7[a]	16.8[a]	13.9[a]	1.12	NS
Glutamate	277.1[a]	269.1[a]	268.8[a]	266.4[a]	8.12	NS
Glutamine	99.2[a]	115.0[a]	121.1[a]	98.6[a]	17.30	NS
Glycine	669.1[a]	930.7[a]	905.2[a]	837.0[a]	121.09	NS
Proline	112.4[b]	149.9[ab]	174.5[a]	135.5[ab]	12.20	0.047
Serine	102.9[a]	118.6[a]	119.2[a]	101.7[a]	11.57	NS
Plasma Urea-N (mM)[2]	2.54[d]	5.07[c]	8.55[b]	11.85[a]	0.464	< 0.001
Urea clearance (ml/min)	18.05[b]	24.80[ab]	32.66[b]	33.15[bc]	2.18	0.003

[1]NS, non-significant
[2]Treatment means with different superscripts are significantly different from each other

Chapter 4: Discussion

Most of the earlier studies which measured urea kinetics in ruminants used dietary manipulations as treatments; for example increasing levels of intake (Sarraseca *et al*., 1998) or increasing levels of dietary N (Marini and Van Amburgh, 2003). Dietary manipulations can alter rumen metabolism thus affecting the process of urea recycling. The objective of this study was to determine whether the ruminant has some control of urea recycling independent of rumen microbial metabolism.

To address this objective required treatments where urea production by the sheep could be manipulated without having to alter rumen metabolism, for example by feeding, and thus alter directly the rumen environment (eg. ammonia production, changes in rumen pH). Thus, different levels of urea were infused into jugular vein as a N source, and intake of the basal diet contained a low level of protein (7.6% on a DM basis) and intake was kept constant. The low protein diet was preferred as it would keep to a minimum rumen ammonia production which otherwise could alter rumen microbial metabolism and fermentation patterns potentially having a negative effect on urea recycling.

The importance of urea recycling, also referred to as 'the protein regeneration cycle' by earlier researchers, has long been recognized as a major mechanism to ensure adequate N for microbial protein synthesis, especially in ruminants fed low protein or low quality roughage-based diets. The relationship of hepatic urea synthesis to digestible N intake varies from 0.93 for steers and 0.88 for diary cows to 1.61 for sheep when compared across different studies (Lapierre and Lobley, 2001).

In the present study, this relationship ranged from 1.53 for the Control to 2.38 for the highest level of urea infusion (Table 10). Under these conditions, in particular for the sheep data, greater net protein gain or milk production can only be achieved by increasing urea recycling to the rumen for microbial protein synthesis. In the present study, urea entry was 53% greater than digestible N intake for the Control treatment, and indeed for this treatment positive N balance was achieved because 85% of urea synthesized was recycled to the GIT for net anabolism. However, even at the low protein intake for the control treatment, considerable amounts of urea-N were transferred to the GIT but returned as ammonia (44%) to the hepatic urea cycle. Thus, one limitation to the recycling process is the capture of urea-N (ie. ammonia-N) within the rumen. Our knowledge of the factors controlling urea recycling to the GIT and the limitations to its capture in the rumen will help in predicting and improving nutrient utilization by ruminants.

As expected, urea entry rate (hepatic ureagenesis plus infused urea) showed a linear increase with each treatment. For the Control, the relationship ureagenesis:digestible N intake was 1.65:1. Thus, when given the Control treatment, i.e. when the sheep received only the basal low protein diet, the sheep were in positive N balance (1.46 g/d) and this required them to recycle and capture significant amounts of urea in the rumen. Indeed, the fractional recycling rate was greatest with ~81% of urea synthesized by the liver partitioned to the GIT (Figure 14). Such high rates of recycling have been reported by various authors (Kennedy and Milligan, 1978; Archibeque *et al*., 2001; Marini and Van Amburgh, 2003) when the animals were fed below maintenance or when kept on a low quality, low protein diet.

Table 10. Comparison of urea entry rate (ureagenesis) to apparent digestible N intake across the four levels of urea infusion in sheep fed a low protein diet.

	Treatment[1] (g urea/d)				SED	*P*[2]
	0	8	16	24		
Intake (g N/d)	10.67	10.67	10.67	10.67		
Apparent digestibility (%)	29.89[b]	38.04[ab]	40.18[a]	41.30[a]	3.47	0.016
Apparent digestible N (g/d)	3.19[b]	4.06[ab]	4.29[a]	4.41[a]	0.370	0.016
UER[3] (g N/d)	5.05[d]	10.56[c]	16.98[b]	21.78[a]	0.619	< 0.001
Urea infusion (g N/d)	0.16	3.76	7.52	11.28		
Ureagenesis (g N/d)[4]	4.89[b]	6.80[b]	9.46[a]	10.50[a]	0.619	< 0.001
Ureagenesis:Apparent digestible N	1.65[a]	1.75[a]	2.12[a]	2.43[a]	0.257	NS

[1]Treatment means with different superscripts are significantly different from each other
[2]NS, non-significant
[3]UER, urea entry rate
[4] Ureagenesis = UER minus urea infusion rate

However, the high fractional rate of recycling (81%) observed during the Control period fell to 63% at the highest level of urea infusion suggesting that the sheep had already met their requirement for N or that entry into the GIT was limited by other factors (eg. dietary energy intake, tissue transporters, rumen ammonia, pH etc) when N supply is higher. Even with the decrease in fractional transfer to the GIT, the absolute amount of urea recycled increased linearly, thus providing more N to the rumen which could have potentially been used for microbial protein synthesis. Thus, the present results indicate that the ability of ruminants to partition urea-N to GIT is much greater and probably less of a limitation than are the events occurring in rumen environment. For example, when sheep are fed chopped lucerne hay, and given intra ruminal infusions of sucrose (Obara and Dellow, 1993), ruminal pH and ammonia concentrations were reduced, as was plasma urea concentration. In turn, they observed an increase in urea recycling to the rumen. By contrast, when ammonia absorption rate was increased by infusion of three levels of ammonium bicarbonate into the mesenteric vein (Milano *et al.,* 2000), urea transfer to the GIT remained constant at 45-47% of liver production but the absolute transfer increased with ammonia infusion.Thus the treatments adopted in these studies may have resulted in different rumen environments resulting in the opposite effects with respect to the proportion of urea partitioned to GIT.

Amongst the few studies that have quantified urea recycling to the GIT, absolute amounts and the proportion of urea recycled to the rumen is often unpredictable in response to total diet and the content of N in the diet. Sarraseca *et*

al., (1998) observed an increase in absolute recycling with different levels of food intake whereas Marini and Van Amburgh (2003) did not observe a significant difference between treatments when Holstein heifers were given five levels of dietary N. The dietary treatments employed in the latter study provided a surplus of N to the rumen which ultimately led to significant increases in rumen ammonia concentration. In consequence, the amounts of urea recycled to the GIT, the portion of that returning to ornithine-urea cycle and the portion of recycled urea used for absorbed microbial amino acids all reached plateaus by the by the second level of N intake. Thus the partition of urea to the GIT may be differentially regulated according to the nature and quantity of the substrates (N, energy etc) available in the rumen.

In the present study, use of recycled urea-N for absorbed amino acids, when expressed as a percentage of digestible N intake, fell from 44% for the Control to 22% at the highest level of infusion. However, the absolute amounts of recycled urea-N used for microbial protein synthesis continued to increase, albeit at a slower rate, with the increasing levels of urea infusion. These results seem to suggest that the microbes were still able to capture urea-N, but with a much lower efficiency (Figure 13). Thus N balance increased with increasing amounts of urea infusion even though the relative efficiencies of microbial capture decreased, due to the fact that much more urea-N was available for capture with each level of infusion. As demonstration of the increase in capture and use of recycled urea for microbial synthesis and amino acid absortpion, plasma concentrations of the essential amino acids methionine and lysine increased. These amino acids are considered to be limiting in microbial protein for growth and milk production (Fraser *et al*., 1991).

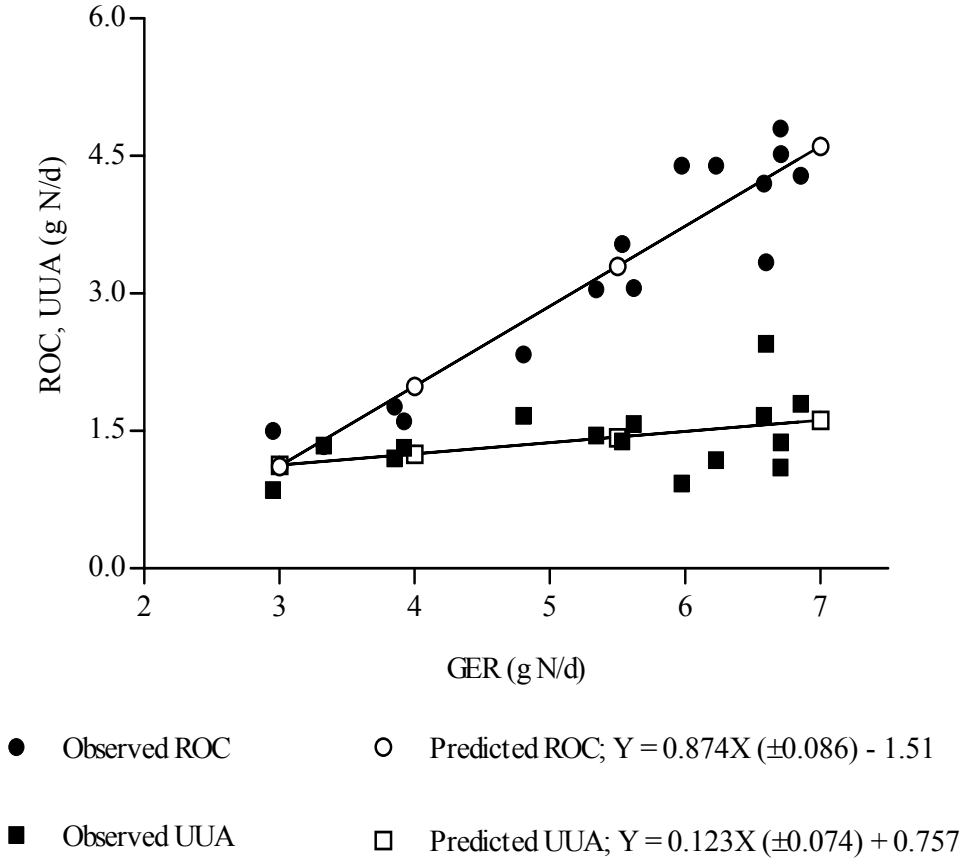


Figure13. Relation ship between ROC to GER and UUA to GER. This graph depicts the relative inefficiency of the microbes to capture and utilize recycled urea-N. The high slope (0.874; $P < 0.001$) for ROC indicates that with increasing levels of urea infusion, more and more urea-N is returned to ornithne cycle avoiding microbial capture. The non significant slope for UUA indicates that with increasing levels of urea infusion, the fraction absorbed as amino acids decreases.

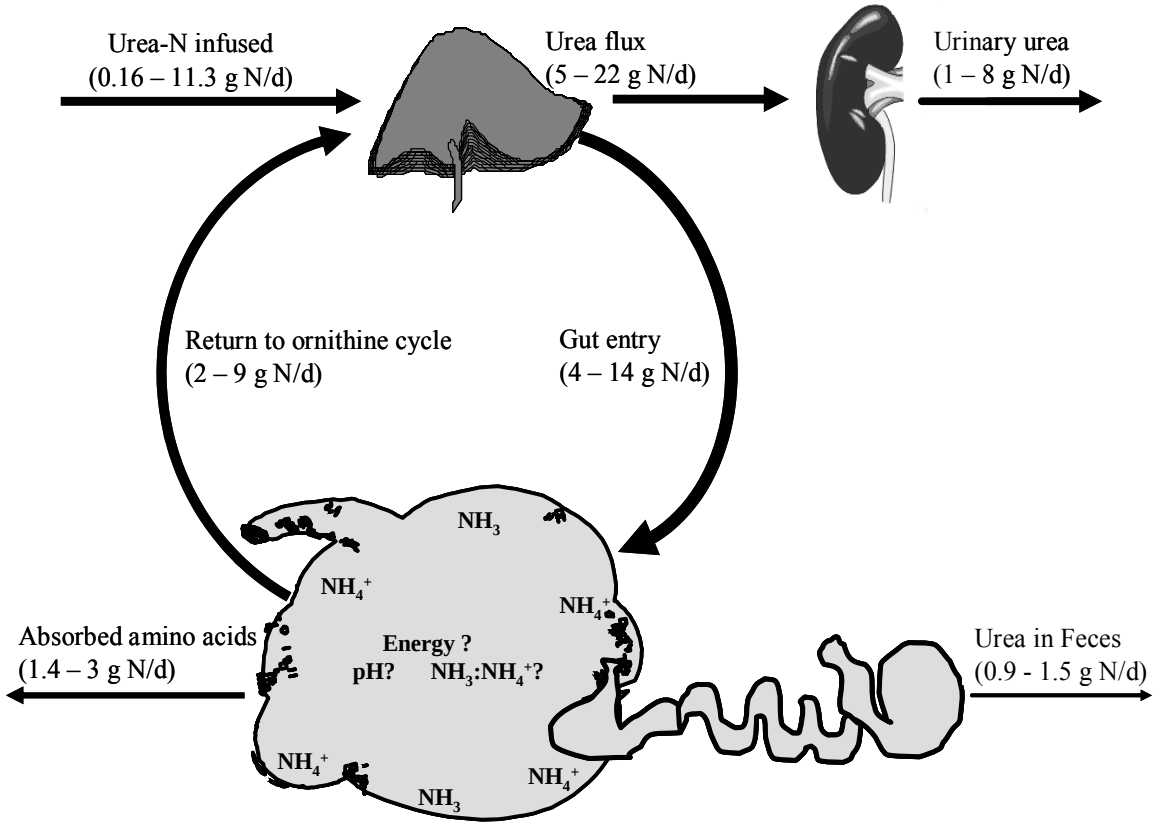


Figure 14. Urea-N flows in sheep in response to four different levels of urea infusion into the jugular vein. With increasing levels of urea infusion, urea flux increased from 5-22 g urea-N/d. Of this 4-14 g urea-N entered the GIT and the rest was excreted in urine. Of the portion partitioned to the gut, 2-9 g urea-N was returned to the liver, 1.4-3 g urea-N absorbed as amino acids and the remained (0.9-1.5 g N/d) excreted in feces.

The concentrations of these two limiting amino acids increased up to the third level of urea infusion, which was the level at which N balance had reached a plateau. The concentrations of all the essential and non essential amino acids declined for the highest level of urea infusion, and this may be the result of gradually correcting for the supplies of the limiting lysine and methionine, in consequence increasing the use of other amino acids for tissue protein deposition. On the other hand, histidine concentrations did not change significantly across the four levels of urea infusion. This might be expected in the case where histidine is the first limiting amino acid. In the study of Fraser et al. (1991), histidine was also singled out as a limiting amino acid in microbial protein.

Even though transfer of urea-N to the GIT remained high at each level of urea infusion, 44-67% of GER returned to the liver as ammonia to be used again for urea synthesis (Figure 14). These values are greater than those reported in other studies (35-42%, Sarraseca *et al*., 1998; 42-51%, Lobley *et al*., 2000; 26-31%, Archibeque *et al*., 2001; 17-35%, Marini and Van Amburgh, 2003). The higher proportional return of urea-derived ammonia could be due to the nature of treatments adopted. Herein, only N availability to the rumen was increased, without a corresponding increase in rumen energy supply via the diet. In consequence, a large proportion of the ammonia formed in the rumen may be in the unionized (NH_4) form which is freely diffusible across the rumen wall, compared to the ionized form (NH_3^+). Since the basal diet we used was forage-based (wheat straw, 36%), the fermentable energy content (7 MJ ME/kg) was much lower than our predictions (10 MJ ME/kg) for growing sheep. In

consequence, the energy content of the diet may have been insufficient to meet the requirements of the microbes for efficient microbial protein synthesis, even on the Control treatment. Provision of additional energy or fermentable carbohydrates would be predicted to decrease the return of urea-derived ammonia to the liver or increase the transfer of recycled urea-N to the rumen, thus further improving N retention (Reynolds and Huntington, 1988). High forage:concentrate ratio diets also affect ammonia production and partition of recycled urea to rumen, small intestine and hind gut, with a higher proportion of urea entry transferred to the small intestine and hind gut (Reynolds and Huntington, 1988; Huntington, 1989). When urea-N is transferred to the small intestines, rather than to the rumen, almost certainly most of the urea will be hydrolyzed, returned to the liver, and reduce the opportunities for rumen microbial capture. This may have occurred in the present study, and contributed to the high values for ROC.

Only small amounts of urea-N were excreted in feces. The fraction of urea-N transferred to the feces was highest (21%) with the Control, decreasing to 11% at the highest level of urea infusion. Fecal N excretion also decreased with increasing amounts of urea infused. This may suggest a progressive increase in the transfer of urea to the rumen rather than to small intestine or hind gut. The increase in N digestibility observed with increasing levels of urea infusions may have occurred as a result of this increase in transfer of urea to the rumen. Hind gut usage of urea-N for microbial protein synthesis represents a loss of N as evidence suggests that there is no absorption of amino acids by the hindgut compartments (Mosenthin *et al.*, 1992).

The extent that urea recycling to the GIT is dependent or 'driven' by plasma urea concentrations has been a subject of debate. Plasma urea concentration has been shown to have low correlations with the amount of urea-N recycled to the GIT (Kennedy and Milligan, 1980; Bunting *et al*., 1987; Lobley *et al*., 1998). Using a larger database, Lapierre and Lobley (2001) also regressed plasma urea-N concentration against net PDV urea-N transfers, based on various studies conducted in sheep and cattle, and found the correlations to be very low ($r^2 < 0.2$) as well. By constrast, we observed significant correlations between plasma urea-N concentration and the amount of urea-N recycled to the gut ($r^2 = 0.92$; Figure 15). The correlations reported previously from databases, included studies where N or total food intake were altered. In the current study, however only plasma urea was altered. In this respect, in the absence of the counteractive effects of diet intake, our data indicate that plasma urea-N is a determinant of urea recycling to the GIT. Failure of the previous analyses to establish high correlations could be explained by an inhibitory effect of rumen ammonia on GIT urea transfer. Thus, as ammonia concentration in the rumen is highly correlated with plasma urea concentration (Kennedy and Milligan, 1978), the effectiveness of plasma urea-N concentration in driving the partition urea-N to the rumen may be negated by the increase in ammonia concentration in the rumen. Situations where this probably occur would included increased CP intake and feeding of a high forage to concentrate ratio diet. This certainly seemed to be the case in this study where, when the body urea pool was manipulated by infusing different levels of urea into the blood, keeping ammonia production in the rumen low, urea recycling to the GIT increased in a linear fashion.

The kidneys may also play an important role in the regulation of urea recycling as evidenced by the increased urea clearance rates with each level of urea infusion. Our observation is consistent with that of Marini and Van Amburgh (2003) where urea clearance decreased in the heifers at the lower N intakes. The importance of the kidneys relative to the GIT may also be important in determining recycled urea-N availability to microbes for microbial protein synthesis.

The results of our study suggest that ruminants have a very high capacity to recycle urea-N to the GIT. That N balance and GER increased with urea infusion, yet ROC also increased, suggests that there may be continuous 'slippage' of rumen urea-N capture. Thus, it may be the rumen environment which is the limiting factor in the capture of recycled N and its utilization for anabolic purposes.

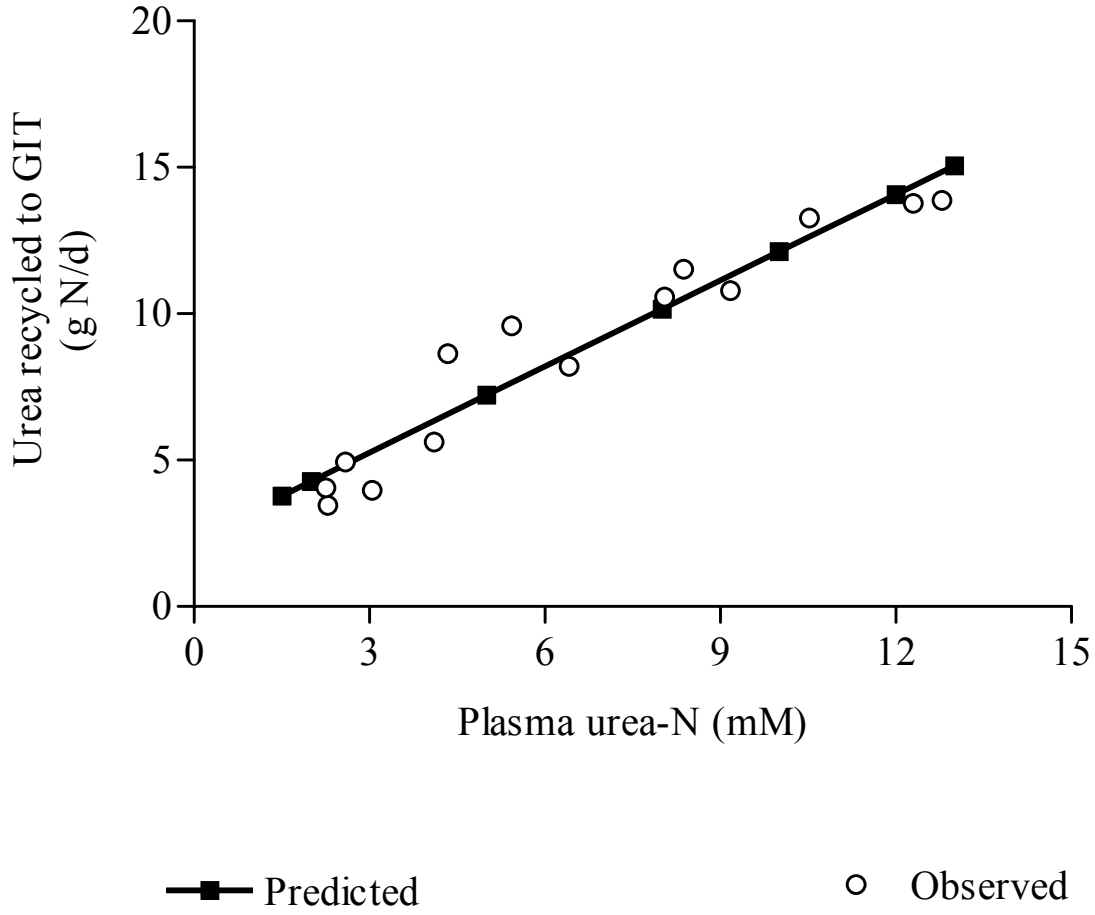


Figure 15. Urea recycled to the GIT (g N/d; y) versus plasma urea-N concentration (mM; x) in sheep fed a low protein diet and infused with increasing levels of urea into the blood.
Regression equation is $y = 0.981x (\pm 0.084) + 2.31$; $r^2 = 0.92$

The Future of Urea Recycling

The rumen environment is a complex ecosystem which is subjected to changes with respect to the type, content and the fermentability of the diet it receives. For example, when ruminants are fed on low protein or low quality herbage diets lacking in sufficient dietary energy, the pH of the rumen is relatively high (6.0-6.5) compared to when higher protein and energy diets are fed (5.5-6.0). Lower rumen pH favors formation of unionized rather than ionized ammonia (Figure 16) in the rumen. Unionized ammonia is freely diffusible across the rumen wall. Thus, if the microbes fail to utilize this ammonia for microbial protein synthesis, as may be the case at our higher levels of urea infusion, ammonia will diffuse across the rumen wall into the portal blood more readily, contributing to high rates of ROC. Providing energy to the rumen at this point would be predicted to either shift the transfer of urea-N more towards the rumen or provide necessary fermentable substrates for microbial metabolism and growth. Soluble carbohydrates lower rumen pH to ~5.5 – 6.0, thus shifting the proportion of ionized to unionized ammonia more towards the ionized form (Figure 17). The urea recycled will be rapidly hydrolyzed due to the high urease activity of bacteria adhering to the rumen wall, creating a 'boundary layer effect' from the local build-up of ammonia (Egan *et al*., 1986). Egan et al. (1986) reasoned that the boundary layer effect is one of the major impediments to the entry of urea from the blood to the rumen, in consequence, increasing the fraction of urea entry excreted in the urine.

Another mechanism regulating urea entry to GIT may be the urea transporters which transport urea across rumen wall by either facilitated diffusion or active transport. Marini and Van Amburgh (2003) demonstrated an increased expression of urea transporters when animals were fed high N diets where as they could not detect any transporter expression when N content in the diet was low. The relative importance of this mechanism may, however, depend on N availability, energy content of diet and rumen environment. Thus urea transport may be primarily occurring through diffusion across the rumen wall in a N limiting environment and may be regulated by urea transporters when N is in excess. This requires further clarification.

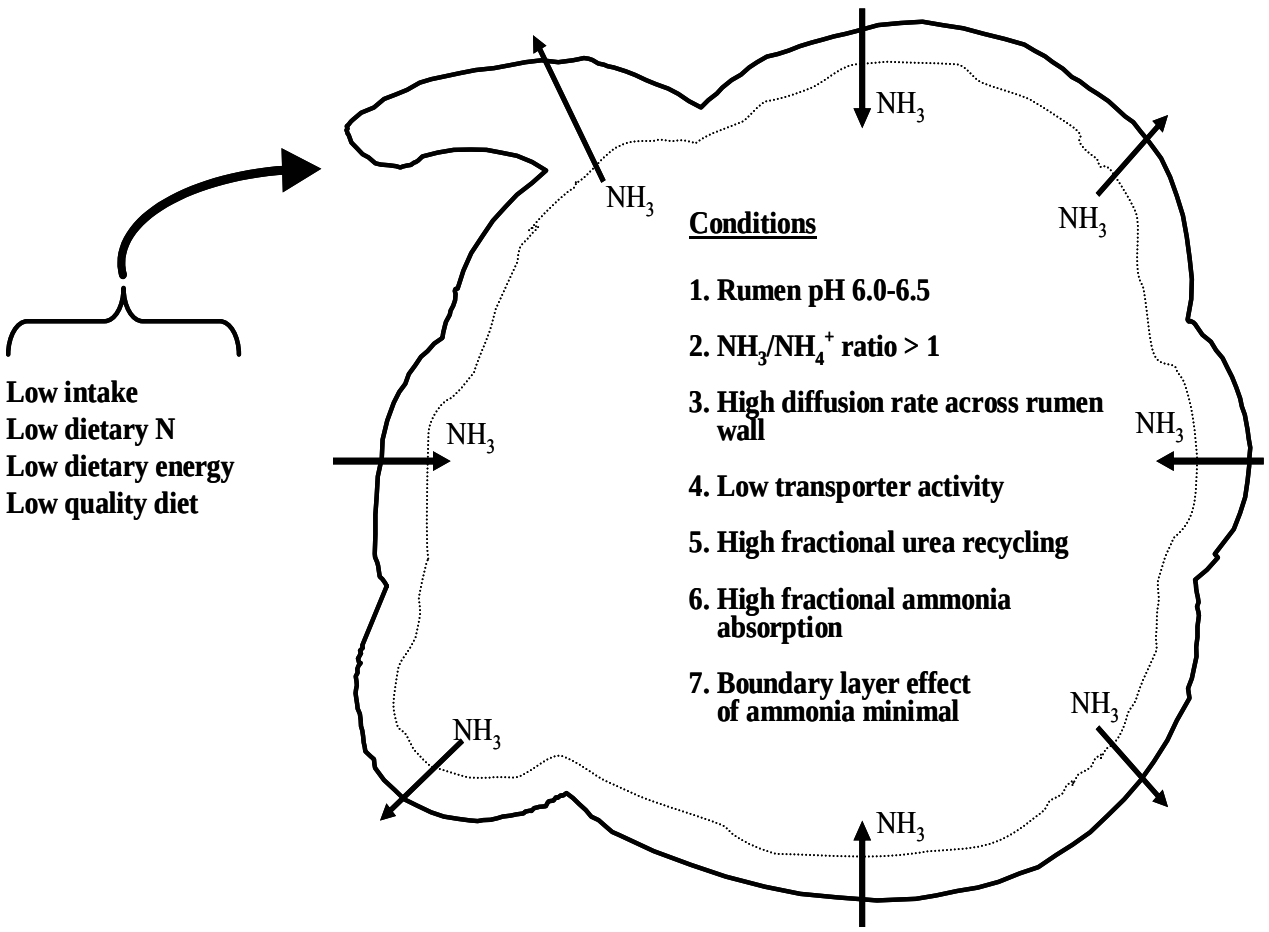


Figure 16. Schematic depicting the conditions that may prevail in the rumen of an animal fed a diet limiting in N and energy. At a relatively higher pH, unionized ammonia predominates, which can diffuse freely across the rumen wall. Thus, such an environment in the rumen may lead to a high rate of urea recycling and also a high rate of ammonia absorption.

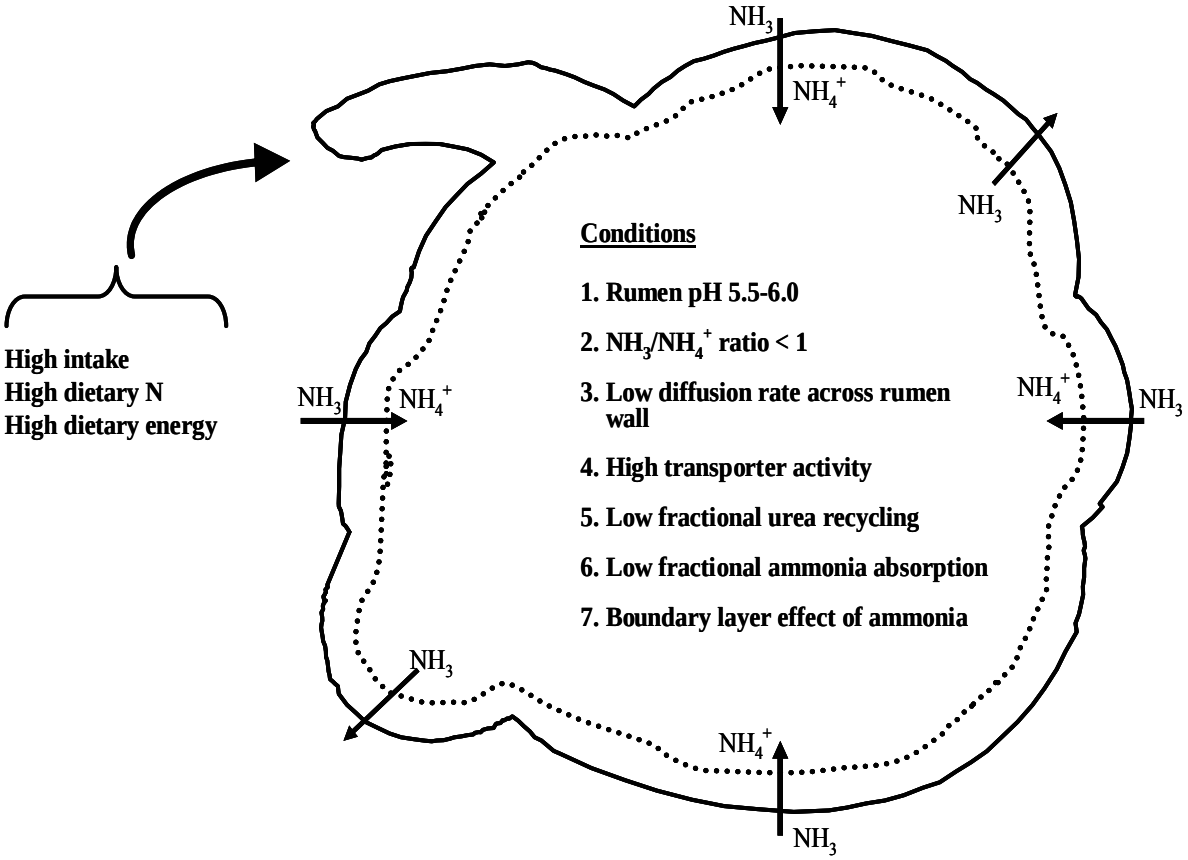


Figure 17. Schematic depicting the conditions that may prevail in the rumen of an animal fed a diet adequate or excess in N and energy. A relatively lower pH in the rumen may contribute to more ionized ammonia than unionized, which gets trapped inside the rumen and cannot diffuse across the rumen wall. Ammonia entering the rumen may also contribute to a 'boundary layer effect' which in turn reduces the rate of recycling. Urea entry to rumen may be regulated more by transporter up regulation rather than diffusion across the rumen wall.

Conclusions

In this study, the ruminant was considered as two compartments, ie. the animal's body and the GIT. An increase in urea production was simulated by infusing increasing amounts of urea into the plasma (jugular vein), rather than by increasing dietary N intake which would result in manipulations to the rumen environment. This approach was taken to test our hypothesis that the ruminant animal, independent of GIT (rumen, intestinal) metabolism, has the ability to self regulate the extent that blood urea is recycled to the GIT for increased microbial protein synthesis, and hence increased amino acids for productive functions (eg. Growth, milk synthesis, wool). The results from this study suggest that the animal in fact has the 'anabolic drive' to recycle urea to the GIT when this process is not constrained by changes or events in the rumen compartment. As a result, very high rates and amounts of urea were recycled to GIT at each level of urea infusion. However, most of the recycled urea failed to be utilized in the rumen for microbial protein synthesis, and instead was progressively reabsorbed as ammonia and converted to urea by the liver. Plasma urea concentration was found to be a major determinant of the partition of urea to GIT, especially in these sheep fed on a low protein diet. Thus, future efforts aimed towards improving N utilization by ruminants would yield more significant results by focussing on rumen dynamics and the factors that could potentially be limiting in the rumen for utilization of recycled N by the microbes. Dietary energy to protein ratio, ammonia concentration in the rumen, the form of ammonia (ionized to unionized) in the rumen, and related to this rumen pH, to name a few, would be some of the factors that will need to be investigated for their role

in urea transfer and capture in the rumen. In this regard, synchronizing the rumen fermentation pattern by careful manipulation of these factors should help to improve N efficiency by ruminants, which in turn should reduce N waste pollution attributed to ruminant production systems.

Implications

Urea recycling to the GIT through plasma, endogenous secretions and saliva is a major contributor towards N retention and the amino acids absorbed by the animal. Urea recycling is of particular importance to the ruminant with 10-80 % of the urea produced in the liver partitioned to the GIT. Of these 30 to 50 % is used by the microbes for microbial protein synthesis in the rumen which is absorbed from the small intestine and utilized for anabolic purposes. Our knowledge of the mechanisms regulating urea partition to the GIT is limited to observations that higher levels of food and protein intake enhance recycling. The results of this study suggest that the ruminant animal has the ability to control partition of urea back to GIT and thus if the rumen environment is manipulated by careful dietary regimes, we can optimize the capture and utilization of recycled urea. Thus, an understanding of the conditions and mechanisms underlying urea partition to the gut will enable us to prepare specific nutrient models for maximizing N efficiency. This knowledge may also help to compensate to a certain extend, the disproportionate losses of essential amino acids occurring due to extensive metabolism by the gut tissues and also due to the endogenous losses.

References

1. Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2000a. Nitrogen metabolism of beef steers fed endophyte-free fescue hay: Effects of rumen protected methionine supplementation. *J. Anim. Sci.* 78(Suppl. 1):302.

2. Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2000b. Urea flux in beef steers: Effects of forage species and fertilization. *J. Anim. Sci.* 78(Suppl. 1):282.

3. Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2001. Urea flux in beef steers: Effects of forage species and fertilization. *J. Anim. Sci.* 79:193-1943.

4. Benlamlih, S, and H. de Pomyers.1989. Changes in endogenous urea recycling and handling of renal urea in pregnant and lactating Sardi sheep kept on a constant feeding level. *Reprod. Nutr. Develop. 29:129-137.*

5. Bergner, H., A. Sommer, O. Simon, R. Goersch, Z. Ceresnakova, L. Chrastinova, J. Szakaos, and M. Stoyke. 1986b. Urea utilization in the large intestine of bulls. *Arch. Anim. Nutr*.36:216.

6. Brockman, R. P. and Laarveld, B. 1986. Hormonal regulation of metabolism in ruminants; a review. *Livest. Prod. Sci*., 14: 313-334.

7. Bunting, L. D., J. A. Boling, C. T. MacKown, and R. B. Muntifering. 1987. Effect of dietary protein level on nitrogen metabolism in lambs: Studies using 15N-nitrogen. *J. Anim. Sci.* 64:855-867.

8. Clark, J. H., Klusmeyer, T. H, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy. Sci. 75:2304-2323.

9. Dixon, R.M., and L. P. Milligan. 1984. Nitrogen kinetics in the large intestine of sheep given Bromegrass pellets. *Can. J. Anim. Sci.* 64: 103-111.

10. Egan, A. R., K. Boda, and J. Varady. 1986. Regulation of nitrogen metabolism an recycling. In Control of Digestion and Metabolism in Ruminants, pp. 386-402 L. P. Milligan, W. L. Grovum and A. Dobson, eds., Prentice-Hall, Englewood Cliffs, NJ.

11. Fraser, D. L., E. R. Orskov, F. G. Whitelaw, and M. F. Franklin. 1991. Limiting amino acids in dairy cows given casein as the sole source of protein. *Livestock Production Science*, 28:235-252.

12. Haussinger, D. 1990. Nitrogen metabolism in liver: structural and functional organization and physiological relevance. *Biochem. J.* 267, 281-290.

13. Hinderer, S. and W. Engelhardt. 1976. Entry of blood urea into the rumen of llama. In tracer studies on non-protein nitrogen for ruminants. III. International atomic energy agency,Vienna. Pages 59-60.

14. Huntington, G. B. 1989. Hepatic urea synthesis and site and rate of urea removal from blood of beef steers fed alfalfa hay or a high concentrate diet. *Can. J. Anim. Sci*. 69:215-223.

15. Huntington, G. B. 1997. Starch utilization by ruminants: from basics to the bunk. *J. Anim. Sci*. 75: 852-867.

16. Isozaki, T., A. G. Gillin, C. E. Swanson, and J. M. Sands. 1994. Protein restriction sequentially induces new urea transport processes in rat initial IMCD. Am. J. Physiol. 266:F756–F761.

17. Jackson A. A., D. Picou and J.P. Landman.1984. The non-invasive measurement of urea kinetics in normal man by a constant infusion of 15N15N-urea. *Cl. Nutr.* 38C, 339-354.

18. Jackson A. A., M. S. Danielsen, and S. Boyes. 1993. A non-invasive method for measuring urea kinetics with a single dose of $^{15}N^{15}N$-urea in free-living humans. J. Nutr. 123, 2129-2136.

19. Kennedy, P. M. 1980. The effects of dietary sucrose and concentrations of plasma urea and rumen ammonia on the degradation of urea in the gastrointestinal tract of cattle. *Br. J. Nutr.* 43:125-140.

20. Kennedy, P. M., and L. P. Milligan. 1978. Transfer of urea from the blood to the rumen of sheep. *Br. J. Nutr.* 40:149-154.

21. Kennedy, P.M., and L. P. Milligan. 1980 The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants: a review. *Can. J. Anim. Sci*. 60, 205-221.

22. Kim, H-S., J. J. Choung, D. Dhamberlain, and G. E. Lobley. 1999. Effect of propionate on ovine urea kinetics. Pages 57 *in* VIIIth International Sym-posium on Protein Metabolism and Nutrition Aberdeen, U. K. Eds. G. Lobley, A. White and J. C. MacRae, Aberdeen United Kingdom, Wagen-ingen Press.

23. Koenig, K. M., C. J. Newbold, F. M. McIntosh, and L. M. Rode. 2000. Effects of protozoa on bacterial nitrogen recycling in the rumen. *J. Anim. Sci.* 78:2431-2445.

24. Lapierre, H., and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: a review. *J. Dairy Sci.* 84(E. Suppl.):E223-E236.

25. Leng, R. A., and J. V. Nolan. 1984. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 67: 1072-1089.

26. Lobley, G. E., A. Connell, M. A. Lomax, D. S. Brown, E. Milne, A. G. Calder, and D. A.H. Farningham. 1995. Hepatic detoxification of ammonia in the ovine liver: Possible consequences for amino acid catabo-lism. *Br. J. Nutr.* 73:667-685.

27. Lobley, G. E., and G. D. Milano. 1997. Regulation of hepatic nitrogen metabolism in ruminants. *Proceedings of the Nutrition Society.* 56:547-563.

28. Lobley, G. E., D. Bremner, R. Nieto, T. Obitsu, A. Hotston Moore, and D. S. Brown. 1998. Transfers of N-metabolites across the ovine liver in response to short-term infusion of an amino acid mixture into the mesenteric vein. Br. J. Nutr. 80:371-379.

29. Lobley, G. E., D. M. Bremner, and G. Zuur. 2000. Effects of diet quality on urea fates in sheep as assessed by refined, non-invasive [15N15N] urea kinetics. *Br. J. Nutr.* 84:459-468.

30. Luo, Q. J., S. A. Maltby, G. E. Lobley, A. G. Calder, and M. A. Lomax. 1995. The effect of amino acids on the metabolic fate of $^{15}NH_4Cl$ in isolated sheep hepatocytes. *Eur. J. Biochem.* 228, 912-917.

31. Marini, J. C., and M. E. Van Amburgh. 2003. Nitrogen metabolism and recycling in Holstein heifers. *J. Anim. Sci.* 2003. 81:545-552.

32. MacRae, J. C., L. A. Bruce, D. S. Brown, D. A. H. Farningham, and M. F. Franklin. 1997b. Absorption of amino acids from the intestine and their net flux across the mesenteric and portal drained viscera of lambs. *J. Anim. Sci.* 75:3307-3314.

33. Milano, G.D., A. Hotston-Moore, and G. E. Lobley. 2000. Influence of he-patic ammonia removal on ureagenesis, amino acid utilization and energy metabolism in the ovine liver. *Br. J. Nutr.* 83:307-15.

34. Mosenthin, R., W. C. Sauer, and C. F. M. de Lange. 1992. Tracer Studies of Urea Kinetics in growing pigs: I. The effect of intravenous infusion of urea on urea recycling and site of urea secretion into gastrointestinal tract. *J. Anim. Sci.* 70:3458-3466.

35. Obara, Y. and D. W. Dellow. 1993. Effects of intraruminal infusions of urea, sucrose or urea plus sucrose on plasma urea and glucose kinetics in sheep fed chopped Lucerne hay. *Journal of Agricultural Science.* 121: 125-130.

36. Reynolds, C. K and Huntington, G. B. 1988. Partition of portal-drained visceral net flux in beef steers. 1. Blood flow and net flux of oxygen, glucose and nitrogenous compounds across stomach and post-stomach tissues. *Br. J. Nutr.* 60: 539-551.

37. Reynolds, C. K., H. F. Tyrrell, and P. J. Reynolds. 1991. Effects of diet forage-to-concentrate ratio and intake on energy metabolism in growing beef heifers: net nutrient metabolism by visceral tissues. *J. Nutr.* 121: 1004-1015.

38. Reynolds, C. K., Lapierre, H., Tyrrell, H. F., Elasasser, T. H., Staples, R. C., Gaudreau, P., and Paul Brazeau. 1992. Effects of growth hormone-releasing factor and feed intake on energy metabolism in growing beef steers : net nutrient metabolism by portal-drained viscera and liver. *J. Anim. Sci.* 70: 752-763.

39. Reynolds, C. K., D. P. Casper, D. L. Harmon, and C. T. Milton. 1992b. Effect of CP and ME intake on visceral nutrient metabolism in beef steers. *J. Anim. Sci.* 70:315.

40. Ritzhaupt, A., G. Breves, B. Schröder, C. G. Winckler, and S. P. Shirazi-Beechey. 1997. Urea transport in gastrointestinal tract of ruminants: Ef-fect of dietary nitrogen. *Biochem. Soc. Trans.* 25:490S.

41. Ritzhaupt, A., I. S. Wood, A. A. Jackson, B. J. Moran, and S. P. Shirazi-Beechey. 1998. Isolation of a RT-PCR fragment from human colon and sheep rumen RNA with nucleotide sequence similarity to human and rat urea transporter isoforms. *Biochem. Soc. Trans.* 26:40.

42. Sarraseca, A., E. Milne, M. J. Metcalf, and G. E. Lobley. 1998. Urea recycling in sheep: Effects of intake. *Br. J. Nutr.* 79:79-88.

43. Seal, C. J., and D. S. Parker. 1996. The effect of intraruminal propionic-acid infusion on metabolism of mesenteric-drained and portal-drained viscera in growing steers fed a forage diet. 2. Ammonia, urea, amino ac-ids and peptides. *J. Anim. Sci.* 74:245-256.

44. Shayakul, C., and M. A. Hediger. 2004. The SLC14 gene family of urea transporters. Eur. J. Physiol. 447: 603-609.

45. Theurer, C. B., G. B. Huntington, J. T. Huber, R. S. Swingle, and J. A. Moore. 2002. Net absorption and utilization of nitrogenous compounds across ruminal, intestinal, and hepatic tissues of growing beef steers fed dry-rolled or steam-flaked sorghum grain. *J. Anim. Sci.* 80:525-532.

46. Wallace, R. J., K. J. Cheng, D. Dinsdale, and E. R. Orskov,. 1979. An independent microbial flora of the epithelium and its role in the ecomicrobiology of the rumen. Nature 279:424-426.