ABSTRACT

Title of Document: ROLE OF VOLATILE FATTY ACIDS IN REGULATING NITROGEN UTILIZATION AND UREA NITROGEN RECYCLING IN RUMINANTS.

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Directed By: Associate Professor, Brian J. Bequette, Department of Animal and Avian Sciences

Mechanistic knowledge of urea-N partitioning has the potential to reveal targets that can be manipulated to improve protein efficiency of ruminants, and hence, reduce N excretion to the environment. The objective of this research was to establish the role of rumen volatile fatty acids (VFA), particularly propionate and butyrate, in regulation of N utilization, urea-N recycling and gluconeogenesis in growing lambs. For these studies, sheep were fitted with a rumen cannula and fed a pelleted ration to $\geq 1.5 \times$ maintenance energy intake. Total urine and feces were collected for determination of N balances. In addition, [$^{15}N_2$]urea was infused to determine urea-N kinetics, [$^{13}C_6$]glucose was infused to estimate gluconeogenesis and [ring-D_5]phenylalanine was infused to estimate protein fractional synthesis rate (FSR) of rumen tissue.

The first study was conducted to evaluate the perturbations in rumen VFA profiles as a result of rumen starch infusion and the association of these perturbations to changes in urea-N kinetics and gluconeogenesis. Sheep (n=4) were infused into the rumen with either water (control) or gelatinized starch (100 g/d) for 9-d periods in a balanced

crossover design. The rumen VFA profile was not affected by starch infusion. Fecal N output tended to increase with starch infusion; however, there were no effects on N retention and urinary N excretion. In addition, starch infusion did not alter urea-N entry rate (UER, i.e. synthesis) nor urea-N recycled to the gut (GER); however, starch infusion increased urea-N excreted in feces (UFE). Glucose entry, gluconeogenesis and Cori cycling were increased by starch infusion. The results suggest that under the feeding conditions of this study, starch infusion shifted the elimination of urea-N from urine to feces but this did not lead to an increase in N retention.

Two companion studies were conducted to determine the role of rumen butyrate in urea-N recycling and rumen FSR. In Exp 1, sheep (n=4) were given intra-ruminal infusions of either an electrolyte buffer solution (Con-Buf; control) or butyrate dissolved in the buffer solution (But-Buf). In Exp 2, sheep (n=4) were infused into the rumen with iso-energetic (1 MJ/d) solutions of either sodium acetate (Na-Ac; control) or sodium butyrate (Na-But). Butyrate infusion treatments increased the proportion of rumen butyrate whereas acetate infusion increased rumen acetate. No difference in N retention was observed between treatments in either experiment. In Exp 2, UER was reduced by Na-But compared to the Na-Ac control, thus, a higher proportion of urea-N entering the rumen was utilized for microbial protein synthesis. In Exp 1, although But-Buf infusion increased the FSR of rumen papillae, urea kinetics were not altered. This study is the first to directly assess the role of butyrate in urea-N recycling and effects on rumen papillae protein turnover in growing lambs. Under the conditions in the present studies, butyrate did not affect overall N retention in growing sheep; however, butyrate reduced urea synthesis and altered the distribution of urea-N fluxes.

Lastly, two companion studies were conducted to determine the role of rumen propionate in urea-N recycling and gluconeogenesis. In Exp 1, sheep (n=6) were continuously infused into the rumen with iso-energetic (1 MJ/d) solutions of either Na-Acetate (control) or Na-Propionate for 9-d periods in a balanced crossover design. In Exp 2, a different group of wether sheep (n=5) were fed on an equivalent protein intake basis either a control or Na-propionate supplemented ration. Propionate treatments increased the proportion of rumen propionate in both experiments. In Exp 1, urea kinetics and N retention were not affected by propionate infusion compared to iso-energetic acetate infusion. However, in Exp 2, the propionate diet increased N retention by ~50%, which resulted from reductions in UER (−2.1 g urea-N/d) and UUE (−0.8 g urea-N/d). Glucose entry and gluconeogenesis were increased by propionate treatments. Under the conditions of these studies, higher ruminal propionate did not affect urea-N fluxes to the rumen. The results from this research provide an understanding of the role of individual rumen VFA in N retention and urea-N recycling in ruminants.

ROLE OF VOLATILE FATTY ACIDS IN REGULATING NITROGEN UTILIZATION AND UREA NITROGEN RECYCLING IN RUMINANTS

By

Umang Agarwal

Dissertation submitted to the Faculty of the Graduate School of the
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Advisory Committee:
Associate Professor Brian J. Bequette, Chair
Professor Richard A. Kohn
Dr. Ransom L. Baldwin, VI
Dr. Erin E. Connor
Assistant Professor Ganesh Sriram
Associate Professor Douglas Julin, Dean's representative

DEDICATION

To

Narendra Modi

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AA	amino acid
BCAA	branched-chain amino acids
BW	body weight
CP	crude protein
DM	dry matter
EAA	essential amino acids
FSR	protein fractional synthesis rate
GC-MS	gas chromatography-mass spectrometry
GER	gut entry rate
GIT	gastro-intestinal tract
MDV	mesenteric-drained viscera
ME	maintenance energy
N	nitrogen
OUC	ornithine-urea cycle
PDV	portal-drained viscera
RDP	rumen degradable protein
ROC	return to the ornithine-cycle
RUP	rumen undegradable protein
UER	urea entry rate
UFE	urea-N excreted in feces
UT	urea transporters
UUA	urea used for anabolism
UUE	urinary urea excretion
VFA	volatile fatty acids

INTRODUCTION

In ruminants, a large portion of valuable protein nitrogen from feed is lost as urea in urine, resulting in low nitrogen (N) efficiency. Conserving this N by redirecting urea towards rumen microbial protein synthesis will help reduce animal manure wastes and increase income over feed costs.

A large but often variable (20-80%) proportion of urea synthesized by the ruminant liver is excreted into the urine as waste, whereas the remainder has the potential to be partitioned to the rumen. Endogenous urea entering the rumen is hydrolyzed to ammonia and utilized by microbes for protein synthesis, or it is reabsorbed across the rumen wall and converted to urea by the liver. Several studies have suggested that higher rumen concentrations of certain volatile fatty acids (VFA), especially propionate and butyrate, increase the ruminal influx of urea (Simmons et al., 2009; Kim et al., 1999). Feeding and post-ruminal infusion of propionate have been shown to increase N retention by growing ruminants, possibly through increasing urea-N recycling and(or) gluconeogenesis (Kim et al., 1999; Baldwin et al., 2012). Moreover, in steers fed a concentrate diet, higher ruminal urea transporter (UT-B) expression was found to be associated with higher rumen butyrate levels (Simmons et al., 2009). Increased expression of UT-B by the rumen epithelia may directly affect the entry of plasma urea into the rumen, thus facilitating its conversion into microbial protein. However, there remains a lack of direct evidence of the influence of rumen VFA on urea-N recycling, and the mechanism(s) by which VFA regulate this recycling process remains unclear. If properly understood, it should be possible to efficiently manipulate feed ingredient inputs

to optimize outputs in terms of amino acid (AA) and energy utilization for meat and milk production by ruminants.

Overall aim of research

The aim of this research was to determine the role of rumen VFA in regulation of N utilization and urea-N recycling to the rumen of growing sheep. The overall goal of this project was to improve AA and protein retention in ruminants through a better understanding of the mechanisms that regulate hepatic urea synthesis and recycling of urea-N in ruminants.

Research Hypothesis

The research conducted in this thesis was designed to address two hypotheses:

1. Higher rumen propionate concentration improves N retention by increasing the amount of urea-N recycled to the rumen, and by sparing AA from gluconeogenesis.

2. Higher rumen butyrate concentration improves N retention by promoting urea-N recycling to and capture in the rumen.

Experimental Objectives

A total of five experiments were conducted to investigate the above hypotheses. The first experiment was conducted to determine, by infusion of starch into the rumen of growing sheep, the profile of rumen VFA that is associated with alterations in urea-N

transfer and capture in the rumen, as well as associations with changes in gluconeogenesis (Chapter 2).

Two companion experiments were conducted to investigate the role of rumen butyrate in N utilization, urea-N kinetics and rumen tissue growth (Chapter 3). In the first experiment, rumen butyrate was artificially elevated by constant infusion into the rumen compared to infusion of buffer (control). In the second experiment, iso-energetic infusions of acetate compared to butyrate were used to simulate forage compared to high concentrate fed rumen fermentation profiles, feeding conditions where urea-N recycling differences have been observed.

Lastly, two companion experiments were conducted to determine the role of rumen propionate (Chapter 4). In the first experiment, iso-energetic infusions of acetate and propionate were used to simulate comparisons of high forage and a high concentrate diet in terms of rumen fermentation profiles. In the second experiment, a forage-type diet with or without added propionate was fed to directly determine the role of propionate in regulation of urea-N kinetics under typical feeding conditions where rumen VFA concentration fluctuates.

CHAPTER 1: LITERATURE REVIEW

Importance of animal agriculture in global food production

Animal agriculture is essential for meeting the global food demand of the increasing population. With the global population already having exceeded 7 billion in 2012, and estimated to exceed 9 billion by 2050 (UN, 2012), this demand is expected to grow even further. Compared to food of plant origin, food of animal origin (meat, milk and eggs) is of high quality and more bio-available to humans in terms of meeting the requirements of essential amino acids (AA), minerals and vitamins. Meat, milk and eggs together account for approximately 17% of global human dietary energy and 35% of human dietary protein consumption (Bradford et al., 1999). Hence, it is important to view animal agriculture as an integral part of a food-producing system rather than an isolated system competing with humans for food demand. Nonetheless, the issue of resource allocation, which involves dynamics of food-feed competition, for food production needs to be scientifically determined keeping in mind the efficiency and nutritional value of various plant and animal products.

Production of plant based human food generates about 27% by-products that are unsuitable for human consumption (Bradford et al., 1999). Given the ability of ruminants to consume human inedible crop residue, and convert it into high quality food for humans, they have been major companions of humans since early civilization. Ruminants not only provide high quality milk and meat, but also provide draught energy and manure for fertilization of fields.

Although ruminant animals require much less human edible feed material as compared to non-ruminant species, the diets of high producing dairy cows may be comprised of 10-30% cereal grains, which seem to place them in competition with human use. Of course, animal diets vary with region and species. The diet of monogastric animals, mainly pigs and poultry, contains 50-70% human-edible grains. In fact, more than one-third of the global consumption of cereal grains is directed towards use as animal feed (Speedy, 2003). Resource use for production of animal products has been questioned on the basis of low efficiency of conversion and environmental concerns of animal agriculture. Most of the criticism has arisen from allocation of human edible food crops towards use as animal feed. However, most often the reported values fail to consider the human inedible nature of feed stuffs.

On the basis of total human-edible food produced per unit human edible food consumed, the returns from ruminants are much higher and the efficiencies are above 100%. For developed countries, the average conversion efficiency of feed grain to high quality human edible food (kg product/ kg grain consumed) for pork, poultry, sheep or goat meat, and milk is 27%, 45.5%, 125% and 333.3% respectively (Bradford et al., 1999). The food production system efficiency calculations must take into account the crop yields in a given land mass, conversion efficiency of the animal and the food processing losses. For example, in the California central valley a comparison was made between alfalfa, the primary animal feed, and wheat, the most suitable grain for human consumption. The yield of alfalfa was shown to be almost four-fold higher than the yield of wheat. The human edible value of total milk produced from alfalfa fed cows and that of wheat fed cows was compared. The total human edible digestible energy (DE) was

20% higher and the dietary protein was twice as high in milk as compared to that in wheat. Thus, the food production systems must be designed based on the regional crop yields and dietary preferences of the population.

Efficiency of nitrogen utilization in ruminants

Although ruminants are unmatched in their ability to convert human inedible material into high quality human edible products, their gross efficiency of converting dietary N into saleable products (milk and meat) is only about 5-35% (Bequette and Sunny, 2005). The remaining 60-80% of the nitrogen (N) is eliminated into the environment, mainly as urea in urine. This results in low N efficiency and has a considerable impact on the environment. There are environmental concerns over inefficient use of N by ruminants since most of the excreted N contributes to ammonia (NH_3) and nitrous oxide in the atmosphere (Federal Register, 2001). And, N loss from manure to water bodies (runoff) contributes to eutrophication. Therefore, a better understanding of N metabolism in the rumen, particularly with respect to NH_3capture by microbes, is needed.

Digestion in ruminants is initiated in the rumen via microbial activity. Microbial activity largely results in degradation of dietary protein and non-protein N sources to NH_3 and carbon skeletons which are re-incorporated into protein by rumen microbes. Depending upon microbial activity and dietary interactions, the intestinal supply of protein and AA may be lower or higher than the true protein intake. However, microbial protein synthesis is not always able to provide adequate protein to meet the demands of high producing animals. Moreover, protein flow through the rumen may result in

degradation of high quality protein. The use of protein supplementation and rumen undegradable protein are some nutritional strategies employed to overcome these limitations (Santos et al., 1998). Further, excessive protein degradation in the rumen results in generation of NH_3 that is absorbed and converted by the liver to urea.

Inefficiency of use of dietary N by ruminants is mainly due to two factors. First, the microbial activity in the rumen leads to the production and absorption of large amounts of NH_3 across the rumen. The second is the absorptive and post-absorptive use of AA. The net flux of essential amino acids (EAA) across portal-drained viscera (PDV, which represents total gut flux) accounts for about 61% that of mesenteric-drained viscera (MDV, which represents net small intestinal metabolism) fluxes (MacRae et al., 1997), suggesting a net extraction of EAA by the non-mesentric gut. El-Kadi et al. (2006) demonstrated in sheep that increasing levels of casein infused into the small intestines aided marginal recovery of EAA in PDV other than branched-chain amino acids (BCAA). Thus, there is a high cost of gut metabolism, particularly non-mesenteric foregut and hindgut tissues, on EAA absorption which contributes to post-absorptive inefficiency of AA use. MacRae et al. (1995) studied the post-absorptive efficiency of AA use in growing lambs fed either forage or forage:barley pellets. Efficiency of utilization of total AA was 50% and 59% with forage and forage:barley respectively.

Urea, the end product of N metabolism, is an important N-dense currency in the overall N economy. At the beginning of the twentieth century, it was found that urea could replace a portion of protein in ruminant rations and by the mid-1950s urea became a well accepted replacement for dietary protein in regions where plant-based protein was limited (Loosli and McDonald, 1968), or simply because urea is cheaper. Since then,

there has been a continuous effort to understand the mechanisms by which urea is manufactured, utilized for anabolic use, or excreted from the body. Conserving N by redirecting urea towards rumen microbial protein synthesis will help reduce animal manure wastes and increase income over feed costs. The cost of ration is the most significant contributor to overall costs of animal agriculture and proteins are an important component of this ration (Beever and Doyle, 2007). Improving the ruminant animal efficiency in terms of N utilization, therefore, will be highly profitable to farmers.

Urea synthesis and metabolic flexibity in the liver

Catabolism of nitrogenous compounds generates NH_3. NH_3 produced in the gastro-intestinal tract (GIT) and peripheral tissue is transferred to the liver to be detoxified to urea by the ornithine-urea cycle (OUC) which comprises five enzymatic steps. The liver is very efficient in extracting NH_3, with efficiency ranging from 75-85% (Lobley and Milano, 1997). However, NH_3 escaping the liver may lead to higher levels of NH_3 in the blood and can lead to toxicity.

Aspartate and glutamate formed by transamination reactions contribute amino acid-N to urea synthesis (Reynolds, 1992). Inputs of cytosolic aspartate and mitochondrial NH_3 are required to “balance” N inputs into the OUC. Lobley et al. (1995) observed that NH_3 removal by the liver in cattle accounted for only 50-60% of urea-N output by the liver. Thus, the remainder most likely derived from catabolism of AA, thus perhaps incurring a penalty on AA supply for tissue protein synthesis in order to balance N inputs to the OUC. Haüssinger (1990) reviewed the 'metabolic zonation' and 'hepatocyte heterogeneity' in the liver cells. As the blood flows from periportal to

perivenous hepatic cells, blood NH_3 is preferentially removed and detoxified to urea. Periportal hepatocytes have a low affinity and high capacity for urea synthesis and the high activity of glutaminase serves as an 'NH_3 amplification system'. On the other hand, the perivenous hepatocytes have a high capacity for glutamine synthesis which serves as a scavenging mechanism to limit NH_3 entry into the peripheral blood. In the liver, NH_3 may also yield aspartate by the coupled reaction of glutamate dehydrogenase and 2-oxoglutarate transaminase (Cohen and Kuda, 1996). These systems provide metabolic flexibility for the N supply to OUC when N inputs are imbalanced. Luo et al. (1995) incubated sheep hepatocytes in $^{15}NH_3$ in the absence of AA and found that [$^{15}N_2$]urea was the major product, thus confirming that both N atoms in urea can derive from NH_3. Analyzing a range of studies in sheep where NH_3 overload was compared to urea-N output, Lobley and Milano (1997) concluded that NH_3 alone contributes approximately 90% of the additional urea-N produced. Thus, enhanced ureagenesis with higher NH_3 absorption incurs a small cost in terms of AA catabolism.

The direct regulation of the urea cycle occurs in the very first step catalyzed by carbamoyl phosphate synthetase I, which in turn is allosterically regulated by N-acetyl glutamate (NAG). NAG synthesis is catalyzed by NAG synthetase and occurs in the mitochondria. Regulation of NAG synthetase occurs by hormones and metabolites. Of particular interest among various regulators is propionate which inhibits NAG synthesis (Stewart and Walser, 1980). However, *in vivo* evidence of inhibition of OUC by propionate in the ruminant liver is lacking. OUC activity has also been shown to be present in gut tissues (Wu, 1995; Oba et al., 2004).

Urea-N recycling in ruminants

Urea synthesized by the liver is released into the blood circulation. Blood urea is either excreted into urine or it is transferred to the GIT compartments primarily by diffusion from blood and saliva (Kennedy and Milligan, 1980). Almost all mammalian systems are capable of recycling urea to the gut. **Table 1.1** summarizes urea-N kinetics in several mammalian species. It is evident that ruminants have an astounding ability to recycle urea synthesized by the liver (urea entry rate; UER) towards the gut (gut entry rate; GER), primarily to the rumen. As urea enters the rumen, it is hydrolyzed to NH_3/NH_4^+ by the microbial ureases (Rémond et al., 1996). The NH_3 generated is either utilized by gut bacteria for synthesis of protein, or it is re-absorbed into the PDV and returned to the ornithine-cycle (ROC) (Lapierre and Lobley, 2001). It is clear from the data in **Table 1.1** that UER can exceed digestible N intake. Thus, under these conditions, urea-N recycling to the rumen for microbial protein synthesis is essential to ensure net N gain by ruminants. It can also be seen that 16 to 81% of the urea-N entering the gut may return to the liver, uncaptured by rumen microbes. Compared to monogastric species, the ability of ruminants to partition a high proportion of urea to the GIT (GER:UER = 30 to 99%) is an evolutionary advantage that allows them to conserve urea in times of N deficiency in the wild (Reynolds and Kristensen, 2008). This wide range of urea-N recycling in ruminants suggests opportunities for improvement of N capture in the rumen.

Table 1.1: Urea-N kinetics in various species.

	g N/d								
Species	N intake	Digestible N	N retention	UER	GER	ROC	GER:UER	ROC:GER	Reference
Sheep	29.1	17.3	7.3	22.4	16.6	7.0	0.74	0.42	(1)
Sheep	17.1	11.4	3.0	16.3	9.9	5.1	0.61	0.51	(2)
Sheep	20.3	15.1	5.1	11.8	4.8		0.41		(3)
Dairy cow	653.2	460.5	27.8	483.4	298.3	224.9	0.62	0.75	(4)
Dairy cow	539.5	422.4	43.7	304.5	169.9	138.2	0.56	0.81	(4)
Steers	72.3	46.4	18.1	43.0	31.3	9.5	0.73	0.30	(5)
Steers	128.1	77.2	42.8	78.0	67.8	24.3	0.87	0.36	(6)
Pigs	28.1	25.8	12.4	20.9	9.7		0.46		(7)
Pigs	36.6	34.5	11.4	34.1	21.5		0.63		(7)
Cats	1.7	1.5	0.0	1.1	0.2	0.1	0.16	0.53	(8)
Cats	4.5	4.3	-0.3	3.9	0.5	0.3	0.12	0.59	(8)

UER = urea entry rate; GER = gut entry rate; ROC = return to ornithine cycle

References: (1) Sarraseca et al. (1998); (2) Lobley et al. (2000); (3) Marini et al. (2004); (4) Gozho et al. (2008); (5) Archibeque et al. (2001); (6) Wickersham et al. (2008); (7) Mosenthin et al. (1992); (8) Russell et al. (2000)

Factors affecting urea-N recycling to GIT

Rumen ammonia and plasma urea concentration

Absorption of NH_3 across the rumen wall results in a potential loss of N, whereas urea transfer from plasma to GIT has the potential to be fixed by rumen microbes into protein. The two main processes across the rumen wall which determine the extent of urea-N recycling are discussed below.

Rumen NH_3 concentration: Rumen NH_3 is primarily generated by the metabolism of nitrogenous compounds (AA, urea, nucleic acids, etc.) by the rumen microorganisms. Up to 50% of digested N appears as NH_3 in the hepatic portal vein which the liver detoxifies to urea (Huntington and Archibeque, 1999). Ammonia is lipid soluble and thus is absorbed directly across the rumen wall, whereas hydrophilic NH_4^+ ions require channels or carriers. The pka of NH_3 is ~9, and according to the Henderson-Hasselbach equation, at pH below 7 (i.e. typical rumen pH) ≥99% of rumen NH_3 will be in the ionized (NH_4^+) form. Therefore, rumen pH is the primary determinant of N exchanges from the rumen to plasma (Abdoun et al., 2010). Absorption of NH_3 across the rumen is linearly related to rumen NH_3 concentration (Abdoun et al., 2005). Dietary protein characteristics, in particular rumen degradable (RDP) or undegradable (RUP) protein, also influences the rate of NH_3 generation in and absorption from the rumen. Rémond et al. (2009) fed a raw pea (high RDP) and extruded pea (low RDP) diet to sheep and observed that the low RDP diet led to a 23% reduction in net absorption of NH_3 by the PDV. Other than level and quality of CP intake, availability of energy substrates in the rumen influence rumen NH_3 concentration. Chamberlain et al. (1985) found that

carbohydrate (glucose, starch and barley) supplementation of a grass-silage diet fed to sheep reduced rumen NH_3 concentration and this was also associated with decrease in rumen pH.

Plasma urea concentration: Transfer of urea from plasma to the rumen is highly dependent upon plasma urea-N concentration. Sunny et al. (2007) demonstrated by intra-venous infusion of urea in sheep fed a low protein diet that urea transfer to the GIT increased in relationship with increases in plasma urea. However, 44-67% of the urea-N entering the rumen returned to the liver as absorbed NH_3 across the levels of urea infused. These results were consistent with Lapierre and Lobley (2001), who suggested that the positive correlation between plasma urea-N and GER is valid for concentrations below 6 mM for sheep and 4 mM for cattle.

Urea transporters

Several studies have reported the presence of facilitative urea transporters (UT) in ruminants but their role in regulating urea transfer to the GIT is not completely understood. The UT derive from two major gene variants, i.e. UT-A and UT-B (Stewart et al., 2005). The presence of UT in the sheep rumen epithelia was first reported by Ritzhaupt et al. (1998). Stewart et al. (2005) characterized the UT-B gene in the bovine rumen tissues and showed that it was expressed in the stratum basale, spinosum and granulosum (inner cell layers) but was absent in the stratum corneum (outer cell layer). Despite the known existence of UT in rumen epithelia, there still remains insufficient evidence of the role of these UT and the nutritional factors that regulate their expression and function. For example, Marini et al. (2004) and Røjen et al. (2011) found no change

in UT protein abundance in rumen tissues of lambs fed increasing levels of dietary protein. Similarly, UT-B mRNA expression was not responsive to changes in dietary RDP (Ludden et al., 2009). Doranalli et al. (2011) observed higher N retention in lambs fed oscillating dietary crude protein (CP) as compared to a medium CP diet. However, there were no differences in phloretin (UT-B inhibitor) sensitive urea flux across rumen tissues employing Ussing chambers. By contrast, Simmons et al. (2009) observed higher UT-B mRNA expression in rumen tissues of steers fed a high concentrate diet compared to steers fed a silage-based diet. However, urea kinetics were not performed and so it remains unclear whether these perturbations in UT-B mRNA expression resulted in greater transfer of plasma urea into the rumen.

Abdoun et al. (2010) conducted an *in vitro* study with sheep rumen epithelium using Ussing chambers. Compared to control tissue, phloretin treatment inhibited urea flux by 50%, demonstrating that urea transport occurs by both diffusion as well as transcellular (UT mediated) mechanisms. Urea transport was found to be pH dependent, with maximum effect at pH 6.2 (bell-shaped curve). Moreover, the effect of pH was only found in the presence of VFA or CO_2. These authors suggested that higher urea transport observed postprandially may be due to acute pH changes in the presence of VFA and CO_2. This may also explain the results obtained by Simmons et al. (2009) where the rumen pH of concentrate-fed steers was 6.15 compared to 6.99 in silage fed steers.

Recently, the role of aquaporins in facilitating urea flux across the ruminal wall has come to light. In Ussing's chamber studies, Walpole et. al. (2013) found that, phloretin-insensitive urea flux (i.e. urea flux on inhibiting UT) across rumen epithelia from calves fed a higher grain diet was elevated compared to a hay based diet. This was

accompanied by higher gene expression of aquaporin. Thus, the role of rumen UT and aquaporins in facilitating urea transport still remains unresolved.

Dietary protein

In general, urea transfer to the GIT increases in parallel with dietary N level, whereas the proportion of urea synthesis transferred to the GIT (GER:UER) decreases. In steers fed increasing dietary N, Archibeque et al. (2001) found that the proportion of N flux absorbed across the rumen was similar, although the efficiency of utilization was greater for lower N intake. The authors found an 11.4% decrease in GER:UER and no change in ROC:GER in steers with high levels of N intake. Marini et al. (2004) observed a quadratic increase in N retention in response to increasing dietary protein intake in lambs, whereas UER and UUE increased linearly.

Fecal N derived from urea transfer to the GIT is fairly constant and thus the main differences occur in urinary N excretion and transfer of urea-N into the rumen. For example, in heifers, the proportion of urea excreted in the urine (UUE:UER) increased from 15 to 71% as dietary CP level increased from 9.7 to 27.2% (Marini and Van Amburgh, 2003).

Dietary protein feeding regime has also been shown to influence N retention in ruminants. It has been demonstrated that feeding oscillating CP diets (i.e. alternating between low and high CP every two days) increased N retention in sheep (Doranalli et al., 2011). The form of protein in the feed (RDP or RUP) also determines N utilization as a result of rates of NH_3 production in the rumen (Kennedy and Milligan, 1980; Lapierre and Lobley, 2001). Rémond et al. (2009) showed that feeding a raw pea (high RDP) diet

to sheep led to greater losses of NH_3 from the rumen compared to feeding an extruded pea (lower RDP) diet. Thus, the amount, frequency and form of dietary CP influences N utilization in ruminants.

Rumen fermentation

About 50 to 80% of the protein flow to the small intestines is derived from microbial protein synthesis in the rumen (Storm and Orskov, 1983). The primary factor which affects rumen fermentation is the fermentability of the diet. Highly fermentable diets increase the amount of urea-N recycled to the rumen (Kennedy and Milligan, 1980; Huntington, 1989) and increase the capture of rumen NH_3 by microbes (Stern and Hoover, 1979), thus leading to lower urinary N losses and hence, greater N retention (Fluharty et al., 1999). Urea synthesis and urinary urea excretion was lower in steers fed starch supplemented gamagrass or orchardgrass based diets (Huntington et al., 2009). In steers, dietary sucrose supplementation has also been shown to enhance the rate of urea transfer to the rumen (Kennedy, 1980). Gozho et al. (2008) studied the effect of barley grain processing on urea kinetics in dairy cows. Rolled barley (highly fermentable) increased GER as compared to pelleted barley (less fermentable), however, the increase in urea-N recycling did not result in greater capture of N in the rumen.

The effect of highly fermentable carbohydrates on urea-N recycling may be due to the changes in rumen VFA profiles. Higher rumen propionate resulting from feeding highly fermentable diets is associated with greater N retention (Abdul–Razzaq and Bickerstaffe, 1989; Obara and Dellow, 1994). On infusion of propionate into the abomasum (i.e. post-rumen) of sheep, Kim et al. (1999) observed an increase in urea

transfer to the rumen. Even when propionate is supplemented to the diet of steers, N retention is increased due to a combination of a reduction in urinary urea excretion and improved dietary N digestibility (Baldwin et al., 2012).

Moloney (1998) observed an interaction between propionate supplementation and feed composition (starch vs. fiber) on N retention in sheep. These authors found that, although higher N retention was observed when feeding a starch-based ration supplemented with propionate, there was no improvement in N retention when a fiber-based ration was supplemented with propionate. In terms of responses to butyrate, Norton et al. (1982) observed an increase in urea recycled to the rumen when sodium butyrate was infused into the rumen, however, N retention was not improved. Thus, whether individual rumen VFA regulate N utilization and urea-N recycling or other factors are more influential remains to be determined.

Production of rumen VFA

In the rumen, VFA are produced by microbial fermentation of energy substrates such as complex carbohydrates (cellulose and hemicelluloses) and starches and pectin derived from plant sources. Although VFA are byproducts of anaerobic microbial fermentation in the rumen, they serve as major energy sources for tissue metabolism. Thus, rumen VFA concentrations depend not only on the macronutrient composition of the feed, but relatedly on the microbial populations and feed retention time in the rumen. Microbial growth is promoted by starch and sugars that are readily fermentable, compared to complex carbohydrates (Stern and Hoover, 1979).

Typical rumen concentrations of VFA vary between 60-150 mM (Bergman, 1990). The molar proportions (mol / 100 mol total VFA) of acetate, propionate and butyrate are typically in the range of 75:15:10 to 40:40:20, depending on the type of diet. Concentrate feeds (cereal grains), which have a high starch content, are rapidly fermented and favor the synthesis of propionate. By contrast, fibrous feeds (forages) lead to greater rumen acetate concentrations. **Table 1.2** compares the effect of various diets containing different concentrate to forage ratios on total rumen VFA concentrations and the molar proportions of acetate, propionate and butyrate. Clearly, including more concentrates in the diet increases the production of total VFA. Moreover, the proportion of rumen propionate and butyrate increase with concentrate diets, leading to changes in rumen VFA profiles.

Metabolic fates of VFA

The VFA produced in the rumen are rapidly absorbed into the blood across the rumen epithelium. Bergman et al. (1965) found that acetate and butyrate are inter-convertible in the rumen by bacteria. Using isotope dilution studies and infusion of radio isotopes of acetate, propionate and butyrate, up to 61% of butyrate carbon and 20% of acetate carbon was shown to arise from acetate and butyrate respectively. Nonetheless, inter-conversion of propionate to acetate or butyrate was found to be negligible. Different VFA, however, have different metabolic fates.

Acetate accounts for 40 to 75% of the total VFA produced in the rumen (**Table 1.2**), and serves as the primary precursor for lipogenesis and energy generation in the body. In lactating ruminants, as much as 25% of fatty acid synthesis in the mammary

glands can be attributed to acetate (King et al., 1985). Annison et al. (1963) studied the metabolism of VFA by infusing radio-isotopes of acetate, propionate and butyrate in the portal vein of sheep. By measuring various blood metabolites they showed that propionate carbons were readily incorporated into glucose, whereas acetate and butyrate did not contribute to net glucose synthesis. Rather, butyrate is mostly converted to β-hydroxybutyrate by the rumen epithelium (Emmanuel, 1980) with butyrate primarily used for energy generation and as a precursor for fatty acid synthesis. Upto ~90% of propionate absorbed across the rumen reaches the liver (Kristensen and Harmon, 2004). The remainder is metabolized by the rumen epithelia, primarily to lactate and CO_2. Since negligible amounts of glucose are absorbed from the small intestines, propionate serves as the most important gluconeogenic precursor in ruminants. The liver metabolizes 80 to 100% of propionate and butyrate absorbed into the PDV, while acetate is largely utilized by peripheral (adipose and muscle) tissues (Bergman and Wolff, 1971).

Table 1.2: Production rates, concentrations and molar proportion of VFA in the rumen of various species. From Dijkstra et al. (2005).

Species	Diet	VFA production (mol/day)	Total VFA (mM)	Acetate (molar %)	Propionate (molar %)	Butyrate (molar %)	Reference
Sheep	Hay	5.8	106	68	19	13	(1)
Sheep	Alfalfa chaff	5.0	131	73	18	9	(2)
Sheep	Alfalfa chaff:corn (1:1)	3.1	73	65	21	14	(2)
Sheep	Alfalfa chaff:corn (1:2)	3.6	113	63	24	13	(2)
Steers	Alfalfa hay:concentrate (4:1)	50.1	103	73	18	9	(3)
Steers	Alfalfa hay:concentrate (1:4)	54.1	108	67	22	12	(3)
Dairy cows	Alfalfa hay:grain (1:1.3)	37.5	109	67	21	12	(4)
Dairy cows	Alfalfa hay:grain (1:6.6)	44.6	121	49	40	11	(4)

(1) Bergman et al. (1965)
(2) Leng and Brett (1966)
(3) Siciliano-Jones and Murphy (1989)
(4) Davis (1967)

Techniques for measuring urea kinetics

The two techniques that are most often used to measure urea kinetics (i.e. rate of urea-N movement across various organs) are the arterio-venous (AV) difference and the labeled urea methods. Using the AV method, net mass transfer of urea across the intestines (MDV), whole gut (PDV) and liver (splanchnic) are directly measured (Huntington, 1989; Rémond et al., 1993; Seal and Parker, 1996). Although there are reports where urea fluxes have been measured under steady-state and non-steady state feeding conditions, the potential errors can be large due to the challenges of accurately measuring AV differencs of urea and matching the AV differences with accurate determination of blood flow. Moreover, this procedure is highly invasive and requires major surgical procedures to introduce catheters, not to mention the challenges of maintaining catheter patency.

In the past two decades, the doubly labeled [$^{15}N_2$]urea method developed by Jackson et al. (1984) has gained much popularity. To date, this is the least invasive method to determine urea kinetics, requiring only an intravenous dose or continuous infusion of labeled urea. This approach allows for measurements of urea production (i.e. synthesis, UER) employing standard isotope dilution principles. Urea-N transfer to the gut (GER) and return of urea-N from gut to the ornithine cycle (ROC) can be estimated from the relative enrichment of urea isotopomers (M1 vs M2) in the urine. This model was adapted for use in ruminants by Sarraseca et al. (1998) and has been previously employed in our lab (Sunny et al., 2007). **Figure 1.1** illustrates the [$^{15}N_2$]urea model.

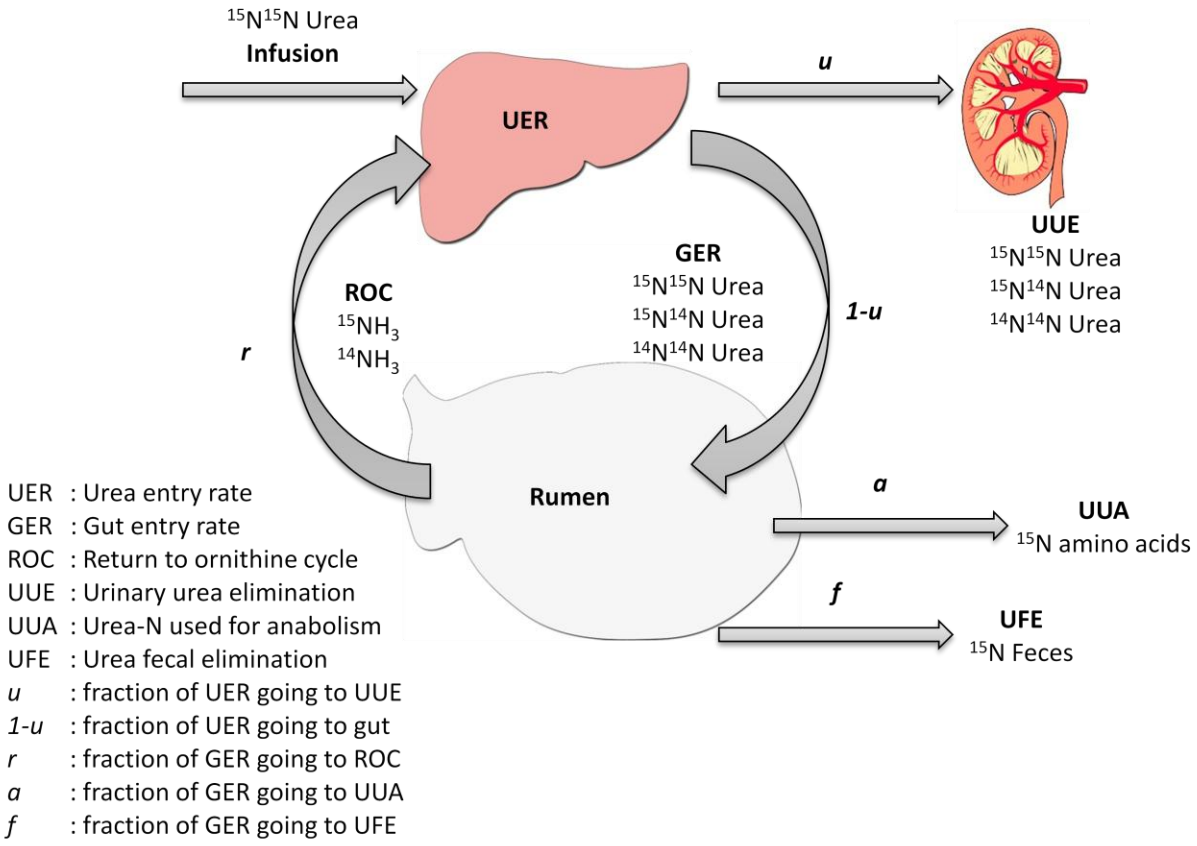


Figure 1.1: Urea-N kinetics model

This is a two compartment model for the flow of urea-N in the body. The first pool is the total body urea-N pool. The entry of urea into this pool arises from synthesis of urea in the liver (UER). The other pool of urea-N is in the gut (GER). When [$^{15}N_2$]urea is continuously infused intravenously over 2 to 5 d, steady-state (plateau) enrichments of various species of urea in the urine are measured. Dilution of [$^{15}N_2$]urea gives an estimate of UER. GER is obtained by the difference of UER and urinary urea excretion (UUE), which is directly measured by total urine collection. It is important to note the GER is not specific to urea-N entering the rumen, but rather the transfer of urea-N to the GIT as a whole. The ratio of [$^{15}N^{14}N$]urea to [$^{15}N^{15}N$]urea reflects the proportion of urea flux

returned to the ornithine cycle (ROC) following bacterial hydrolysis to $^{15}NH_3$.The basic assumption in this model is that the probability of forming [$^{15}N_2$]urea in the liver, i.e. simultaneous incorporation of two molecules of $^{15}NH_3$ into urea, is negligible provided the infusion of [$^{15}N_2$]urea does not exceed 5% of UER. This model avoids overestimation of ROC using a correction factor for multiple entries of [$^{15}N^{14}N$]urea from blood into the gut, based on a geometric decline in return of the recycled ^{15}N label. Utilization of $^{15}NH_3$ for microbial protein synthesis, and subsequent catabolism of the labeled AA, also contributes to the appearance of [$^{15}N^{14}N$]urea, however, this contribution is very small and is assumed to be negligible. Urea-N excreted in feces (UFE) is directly measured from fecal ^{15}N output and, thus, anabolic use (UUA, i.e. microbial use) is calculated by difference. Because UUA is estimated by differences, it's accuracy is subject to cumulative errors. Moreover, steady-state conditions are required and this requires the constant infusion of [$^{15}N_2$]urea for at least 2 d to attain plateau enrichments of the [^{15}N]urea isotopomers and of fecal ^{15}N.. However, the major advantage of employing the [$^{15}N_2$]urea method lies in its ability to estimate urea-N returning from the gut, and thus allow for the estimation of urea-N utilized by gut microbes.

Summary

It is clear from the literature that urea-N recycling and N utilization in ruminants is a complex process. Among the dietary factors, CP and energy supply are most prominent in regulating N utilization. The efficiency of N utilization and recycling of urea to the gut is conversely variable with higher N intake, and there is also a huge variation seen with the quality (rumen degradability) of CP. Other than level and quality of CP intake, energy supply and fermentability of the diet also play a role in N utilization.

There are several reports that suggest that the higher concentration of rumen propionate and (or) butyrate, resulting from highly fermentable diets, influence the regulation of urea-N recycling. However, the role of individual VFA in regulating urea-N kinetics and increased N utilization is not well understood. Thus, based on available literature and results from previous experiments in steers (Baldwin et al., 2012), we hypothesized that higher rumen propionate and(or) butyrate would improve N retention by increasing the amount of urea-N recycled to the rumen.

CHAPTER 2: EFFECT OF RUMINAL STARCH INFUSION ON PERTURBATION OF RUMEN VOLATILE FATTY ACIDS, UREA NITROGEN KINETICS AND GLUCONEOGENESIS IN GROWING SHEEP

Abstract

Starch supplementation is known to increase N retention in growing ruminants, possibly through increasing urea-N recycling to the rumen. This study was conducted to evaluate the perturbations in rumen volatile fatty acid (VFA) profiles as a result of rumen starch infusion and the association of these perturbations to changes in urea-N kinetics and gluconeogenesis in growing sheep. Wether sheep (n=4, 36.9 ± 3.45 kg BW), fitted with a rumen cannula, were fed to 1.5 × maintenance energy intake a pelleted concentrate-type ration (165 g CP/kg, 9.3 MJ ME/kg) and infused into the rumen with either water (control) or starch (gelatinized; 100 g/d) for 9-d periods in a balanced crossover design. [$^{15}N_2$]Urea was continuously infused i.v. for the last 5 d of each period, and total urine collected by vacuum and feces by a harness bag. Over the last 12 h, [$^{13}C_6$]glucose was continuously infused i.v. and hourly blood samples collected during the last 5 h. All animals were in positive N balance (~4.2 g N/d). Rumen VFA concentrations were not affected by starch infusion. Starch infusion reduced ($P < 0.05$) N digestibility (~5%) and tended ($P < 0.1$) to increase fecal N output (5.6 vs 6.4 g N/d), however, there was no effect on N retention or urinary N excretion. In addition, starch infusion did not alter urea-N entry rate (synthesis) or urea-N recycling, however starch infusion did increase ($P < 0.05$) urea-N excreted in feces (+0.9 g urea-N/d) and tended (P

= 0.1) to reduce urinary urea-N excretion (6.6 vs 5.2 g urea-N/d). Glucose entry (128 vs 177 g/d), gluconeogenesis (98 vs 132 g/d), and Cori cycling (30 vs 45 g/d) were increased ($P < 0.05$) by starch infusion. The results suggest that under the feeding conditions of this study, starch infusion shifted the elimination of urea-N from urine to feces but this did not lead to an increase in N retention.

Introduction

Highly fermentable (high starch) diets increase the amount of urea-N recycled to the rumen (Kennedy and Milligan, 1980; Huntington, 1989) and increase the capture of rumen ammonia (NH_3) by microbes (Stern and Hoover, 1979), thus leading to lower urinary N losses and hence, greater N retention (Fluharty et al., 1999). Increasing the level of rumen degradable starch results in higher production of rumen volatile fatty acids (VFA), which are responsible for meeting ~70-80% of the animal's energy needs (Bergman and Wolff, 1971). Starch supplementation of forage-type diets has also been shown to promote microbial growth (Stern and Hoover, 1979). Huntington et al. (2009) found urea synthesis and urinary urea excretion to be reduced in steers fed gamagrass or orchardgrass diets that had been supplemented with starch. And, dietary sucrose supplementation has been shown to enhance the rate of urea transfer to the rumen of steers (Kennedy, 1980).

There are several reports that suggest that the higher concentration of rumen propionate and (or) butyrate, resulting from highly fermentable diets, influence the regulation of urea-N kinetics (Kim et al., 1999; Simmons et al., 2009). For example, Simmons et al. (2009) reported greater rumen epithelial expression of the urea

transporter, UT-B, in steers fed a concentrate compared to a mostly forage-based diet. The higher rumen butyrate concentrations in steers fed the high concentrate diet led these authors to propose that butyrate might be involved in regulation of UT-B expression, and thus urea-N recycling to the rumen. By contrast, Kim et al. (1999) had observed that post-ruminal (abomasal) infusion of propionate in growing sheep increased urea synthesis, and transfer and capture of urea-N in the rumen, thus leading to increased N retention. To that end, it is clear that the role of individual VFA in regulating urea-N kinetics and increased N retention is not well understood.

The aim of this study was to determine, by infusion of starch into the rumen of growing sheep, the profile of rumen VFA that is associated with alterations in urea-N transfer and capture in the rumen, as well as associations with changes in gluconeogenesis.

Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland (protocol number R-11-33).

Animals, diet and treatments

Four Polypay × Dorsett wether lambs (36.9 ± 3.45 kg BW), fitted with a rumen cannula, were fed a standard pelleted diet (**Table 2.1**) to 1.5× maintenance energy (ME) requirements every 2 h via automatic feeder. Sheep were placed into individual metabolic crates and assigned to receive rumen infusions of either starch (100 g/d) or water (control) in a balanced cross-over design with each infusion period lasting 9 d. Starch

infusion rate was calculated to supply 15% additional ME intake (based on gross energy of starch). The starch infusate was prepared by dissolving 100 g of starch in 1 L water, followed by autoclaving to gelatinize the starch. Each infusion period was separated by 5 d during which sheep were placed into individual floor pens for exercise and to allow a period of treatment washout.

Tracer infusion

Temporary jugular vein catheters were inserted at least 2 d prior to initiating isotope infusions. Over the last 4 days of each treatment period a sterile solution containing [$^{15}N_2$]urea (99 atom percent ^{15}N, Cambridge Isotope Laboratories Inc., Andover, MA) was continuously infused (0.6 g/d) through a jugular vein catheter. It has been shown that this length of [$^{15}N_2$]urea infusion results in the attainment of isotopic plateau in urinary [$^{15}N^{14}N$] and [$^{15}N^{15}N$]urea enrichment and that this infusion rate increases the urinary [$^{15}N^{15}N$]urea enrichment to 1.5-2.5 atoms percent excess (APE) (Sunny et al., 2007). Glucose kinetics were measured by infusion of [$^{13}C_6$]glucose. On the last day of each experimental period, an *i.v.* bolus (priming) dose (0.45 g) of [$^{13}C_6$]glucose was administered followed by continuous infusion (0.15 g/h) for 9 h.

Sampling

During the last 5 d of each treatment period, sheep were fitted with a harness for total collection of feces (by bag) and urine (by suction), which were weighed and recorded. A slight vacuum was used to collect urine directly into a sealed container placed on a stir plate and containing sufficient HCl to reduce urine pH to < 3 to prevent bacterial hydrolysis of urea and ammonia volatilization. Well-mixed sub-samples from

each collection (100 g feces and 40 g urine) were stored at -20^{o}C for later analysis. Over the last 6 h of tracer infusion, urine samples were also collected at 2 h intervals and later analyzed to verify that plateau of [$^{15}N_2$]urea enrichment in urine had been attained. Hourly blood samples were collected during the last 5 h. Plasma was separated by centrifugation (1,000 × *g* for 15 min at 4^{o}C) and stored at -20^{o}C for later analysis. At the end of each experimental period, following collection of the last sample, sheep were removed from crates and within 10 mins the rumen was completely evacuated by vacuum. The rumen fluid was mixed well and a representative sample (50 mL) strained through two layers of cheese cloth. The strained rumen fluid was immediately centrifuged (1,000 × *g* for 10 min at 4^{o}C) and the pH of the supernatant recorded using a pH indicator strip (VWR International, West Chester, PA). For determination of VFA concentration, to a known amount (1 g) of rumen fluid was added a known weight (0.25 g) of an internal standard mixture (260 mM [1-^{13}C]acetate, 80 mM [methyl-D_3]propionate and 40 mM [1-^{13}C]butyrate), and the samples stored at -20^{o} C for later analysis.

Table 2.1: Composition of diet.

Item	Amount, g/kg (as fed)
Ingredient	
Ground Corn	385
Alfalfa	540
Soybean Meal	50
Vitamin-mineral premix[1]	25
Chemical composition (g/kg DM)	
DM	877
CP	165
Soluble Protein	41
ADF	164
NDF	226
Starch	351
Crude fat	33
TDN	730
N_{em}, MJ/kg	7.19
N_{eg}, MJ/kg	4.61

[1]Bel Air sheep mineral vitamin mixture (per kg of premix): Ca, 210 g; P, 30 g; NaCl, 160 g; S, 30 g; Mg, 25 g; K, 24 g; Fe, 2.4 g; Mn, 2.4 g; Zn, 2.7 g; Se, 24 mg; Co, 30 mg; I, 40 mg; Choline-Cl, 4.41 g; vitamin A, 661,500 IU; vitamin D_3 132,300 IU; vitamin E, 1,764 IU

DM: dry matter; CP: crude protein; ADF: acid detergent fiber; NDF: neutral detergent fiber; TDN: total digestible nutrients

Urea concentration and enrichment

The concentration and enrichment of plasma and urinary urea were determined by gas chromatography-mass spectrometry (GC-MS, Agilent 6890 series gas chromatography system coupled to an Agilent 5973N mass selective detector; Agilent; Palo Alto, CA) under electron ionization (EI) mode. For determination of urinary urea concentration, to a known weight (0.25 g) of an internal standard solution containing [^{13}C, $^{15}N_2$]urea (5 mg/g) was added an equal known weight of urine. For plasma urea concentration, a known amount (0.5 g) of internal standard containing [^{13}C, $^{15}N_2$]urea (0.26 mg/g) was added to an equal known weight of plasma. Plasma and urine samples were acidified by adding an equal volume of 15% sulfosalicylic acid or 10% trichloroacetic acid (w/v), respectively, and centrifuged for 10 min at 10,000 × *g* to precipitate proteins and other debris. Urea was isolated from acidified samples by application to 0.5 g of cation exchange resin (AG 50W-X8 resin, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA). The resin was washed with 2 × 2 mL of water and urea eluted with 2 mL of ammonium hydroxide plus 1 mL of double distilled water. An aliquot (100 to 200 µl) of the elute was dried under N_2 gas and the tertiary-butyldimethylsilyl derivative of urea was prepared by adding 50 µl each of acetonitrile (Pierce chemicals , Rockford, IL) and N-methyl-N-t-butyl-dimethylsilyl-trifluroacetamide (MTBSTFA, Pierce chemicals , Rockford, IL) followed by heating at 90^{o}C for 20 minutes (El-Kadi et al., 2006). Derivatized urea samples were separated on a fused silica capillary column (HP-50; 30 m × 0.25 mm × 1 µm Hewlett-Packard, Palo Alto, CA) prior to MS under EI conditions. Ions of mass-to-charge (m/z) 231.2 (unlabeled; M0), 232.2 (singly labeled; [M+1]), 233.2 (doubly labeled; [M+2]), and

234.2 (internal standard; [M+3]) were monitored. The GC inlet was set at 250 ^{o}C and the GC conditions were: initial temperature of 150^{o}C followed by 15^{o}C/min to 250^{o}C. Urea concentration in the samples was calculated by isotope dilution (Calder et al., 1999) based on the ratios of [M+3]:[M0]urea after correction for background (natural abundance) and spillover effects of the enriched [M+2]urea.

Total urinary and fecal N, and fecal ^{15}N

Total urinary and fecal N were measured using an automated N analyzer (CN-2000, Leco, St. Joseph, MI). Fecal samples were dried in a forced-air drying oven at 60^{o}C for 5 d. Dried samples were pulverized in a liquid nitrogen freezer mill (Freezer-Mill 6850, Spex CertiPrep Inc., Metuchen, NJ) and samples submitted to the stable isotope facility at the University of California-Davis (CA) for ^{15}N analysis by isotope-ratio mass spectrometry.

Rumen fluid VFA concentration

Samples were thawed and acidified by adding 250 µL of 2 M HCl, and centrifuged for 15 min at 13,000 × *g* to precipitate solids. The supernatant was filtered through a 0.2 µm filter (Nalgene, Rochester, NY), and anhydrous ether (0.5 mL) added to extract VFA. An aliquot (200 µl) of extracted VFA was converted to the tert-butyldimethylsilyl derivative by adding 50 µL MTBSTFA followed by heating at 80^{o}C for 20 min (Duncan et al., 2004). After samples had cooled, 50 µL of methanol was added to react with the surplus MTBSTFA. Derivatized samples were injected onto the GC column (fused silica capillary column, HP-5; 30 m × 0.25 mm × 1 µm Hewlett-Packard) with the GC inlet set at 250°C and the following columns conditions: initial

temperature of 60^{o}C held for 3 min followed by 10^{o}C/min to 210^{o}C for 4 min. Ions of m/z 117 and 118 for acetate, 131 and 134 for propionate and 145 and 146 for butyrate were monitored. VFA concentrations were calculated by isotope dilution (Calder et al., 1999).

Glucose concentration and enrichment

For determination of plasma glucose concentration, a known amount (0.5 g) of an internal standard containing [$^{13}C_6$; 1, 2, 3, 4, 5, 6, 6-$^{2}H_7$]glucose (4 mM in 0.1 M HCl) was added to an equal known weight of plasma. The samples were acidified with an equal volume of 15% sulfosalicylic acid (w/v) and centrifuged for 10 min at 10,000 × *g* to precipitate proteins. The supernatant was applied to 0.5 g cation-exchange resin (AG 50W-X8 resin, 100-200 mesh) and the glucose containing fraction eluted with 2 mL distilled water, frozen and lyophilized to dryness. Glucose was converted to the di-O-isopropylidene derivative for GC-MS analysis (Hachey et al., 1999). Briefly, to the freeze dried sample was added 1 mL of freshly prepared 0.38 M sulfuric acid in acetone. The solution was incubated for 1 h at room temperature after which it was neutralized by the addition of 3 mL of 0.44 M sodium carbonate. After the addition of 3 mL of saturated NaCl, the glucose derivative was back extracted with 3 mL ethyl acetate. The upper ethyl acetate phase was dried under N_2 gas. Next, the di-O-isopropylidene derivative was acetylated by the addition of 50 µL each of ethyl acetate and acetic anhydride followed by heating for 30 min at 60^{o}C. The glucose derivative was separated on a fused silica capillary column (HP-5; 30 m × 0.25 mm × 1 µm, Hewlett-Packard) prior to MS under EI conditions. The GC inlet was set at 250°C and the following columns conditions: initial temperature of 80^{o}C followed by 10^{o}C/min to 260^{o}C. Ions of m/z 287 to 293 (glucose isotopomers [M0] to [M+6]) and 300 (internal standard; [M+13]) were monitored.

Glucose concentration in samples was calculated by isotope dilution (Calder et al., 1999) based on the ratio of [M+13]:[M0]glucose after correction for background (natural abundance), and corrected for the concentration of [M+1]-[M+6]glucose isotopomers. The enrichments of plasma glucose were corrected for natural abundance using a matrix approach (Fernandez et al., 1996).

Calculations

Urea-N kinetics: Urea-N kinetic calculations were based on Lobley et al. (2000). Whole body urea synthesis (urea entry rate; UER) was estimated from the dilution of the infused [$^{15}N_2$]urea tracer. A portion of UER is excreted into urine (UUE), whereas the remainder enters the gut tissues (GER). Urea entering the gut has three different fates: 1) excretion in feces (UFE), 2) hydrolysis by rumen microbes with absorption of NH_3 and return to the ornithine-urea cycle (ROC), and 3) utilization by gut microbes for protein synthesis (anabolic use, UUA). UFE and ROC are estimated directly from excretion of fecal ^{15}N and based on the appearance of urinary [$^{15}N^{14}N$]urea, respectively. UUA is estimated by the difference between GER and UFE+ROC. An important assumption in this model is that all the doubly labeled urea in urine is derived from the infused tracer. Calculations for urea N kinetics are shown in Appendix 3.

*Gluconeogenesis:*Gluconeogenesis and glucose recycling (Cori cycling) were estimated using corrected mass isotopomer distribution (MID) enrichments according to a method described by Tayek and Katz (1997), where:

Glucose entry (g/d) = (([$^{13}C_6$]glucose tracer purity (%) / [M+6]glucose) – 1) × [$^{13}C_6$]glucose infusion rate (g/d), and

Cori cycling (g/d) = ([M+1] + [M+2] + [M+3]) / ([M+1] + [M+2] + [M+3] + [M+6]) × glucose entry (g/d)

Gluconeogenesis, i.e. gluconeogenesis from non-glucose sources, was calculated by difference as,

Gluconeogenesis (g/d) = Glucose entry - Cori cycling

Glucose entry represents the appearance in blood of glucose derived from absorption and from synthesis in the body. However, because dietary starch is extensively fermented by rumen microbes and the fact that nearly all glucose absorbed across the intestines is metabolized, it is safe to assume that net glucose absorption across the gut is negligible (El-Kadi et al., 2006). Thus, glucose entry largely reflects Cori cycling and gluconeogenesis.

Statistical Analysis

Animals were nested within groups. Treatment and period were considered to be fixed effects and animals within group were considered to be random effects. Data were analyzed using the PROC MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). The following linear model was used to analyze the data:

$$Y_{ijk} = \mu + group_i + animal_{ij} + period_k + treatment_h + error_{ijk}$$

where,

Y_{ijk} = response variable (UER, UUE etc.) in the k^{th} period of the j^{th} animal in the i^{th} group (i = 1, 2; j = 1 to n; k = 1, 2)

μ = the overall mean effect

$group_i$ = the effect of the i^{th} group (i = 1,2)

$animal_{ij}$ = the effect of the j^{th} animal on the i^{th} group (j = 1, 2, .. , n_i)

$period_k$ = the effect of the k^{th} period (k = 1,2)

$treatment_h$ = the effect of the h^{th} treatment (h = 1,2; being a function of i & k)

$error_{ijk}$ = the residual error

Statistical significance was set at $P \leq 0.05$ and a trend at $P \leq 0.10$.

Results

Feed intake, digestibility and N-retention: All animals gained weight during the experiment. Dietary DM digestibility (69 to 75%) was not different between treatments, however, starch infusion reduced ($P < 0.05$) N digestibility (**Table 2.2**). Fecal N tended ($P < 0.1$) to increase with starch infusion, however, there was no effect on N retention or urinary N excretion.

Rumen pH and VFA profiles: Despite predictions that starch infusion would increase rumen propionate and(or) butyrate concentrations and molar ratios, the rumen VFA profile and pH were similar between treatments (**Table 2.3**).

Table 2.2: Nitrogen balance in growing sheep (n=4) infused with starch or water (control).

	Water (control)	Starch	SE	*P*
N balances[1] (g N/d)				
N intake	22.93	22.93	0.60	NS
N in urine	13.41	12.13	1.13	NS
N in feces	5.63	6.37	0.58	0.081
N retained	3.90	4.43	0.84	NS
DM digestibility (%)	72.26	70.91	0.97	NS
N digestibility (%)	75.56	72.06	1.33	0.040
Urine urea-N:total urine N	0.50	0.43	0.02	NS
N retained:N digested	0.20	0.28	0.05	NS

[1]Nitrogen balance was measured during the last 4 d of each 9 d treatment period.

Table 2.3: Rumen volatile fatty acid profiles in growing sheep (n=4) infused with starch or water (control).

	Water (control)	Starch	SE	*P*
Concentration (mM)				
Acetate	167.50	172.14	32.99	NS
Propionate	21.93	30.49	4.21	NS
Butyrate	14.03	10.01	2.69	NS
Molar proportion (mol/100 mol)[1]				
Acetate	81.23	81.45	2.05	NS
Propionate	11.69	13.82	2.05	NS
Butyrate	7.07	4.73	0.88	NS
pH	6.13	5.75	0.15	NS

[1] Moles of each VFA relative to the sum of acetate, propionate and butyrate.

Urea-N kinetics: For both treatments, urinary [M+2]urea enrichment reached an isotopic plateau (**Figure 2.1**) within 2 d of [$^{15}N_2$]urea infusion. Urinary [M+1]urea enrichment increased until attaining a plateau between 2 and 3 d of infusion (**Figure 2.2**). Total fecal ^{15}N enrichment continued to increase throughout the infusion period, hence, the fecal ^{15}N enrichment on the last day was used in calculations. Urea-N kinetics are shown in **Table 2.4**. There was no change in UER, however, UUE tended ($P = 0.1$) to decrease and UFE increased ($P < 0.05$) with starch infusion. Plasma urea concentration was not affected.

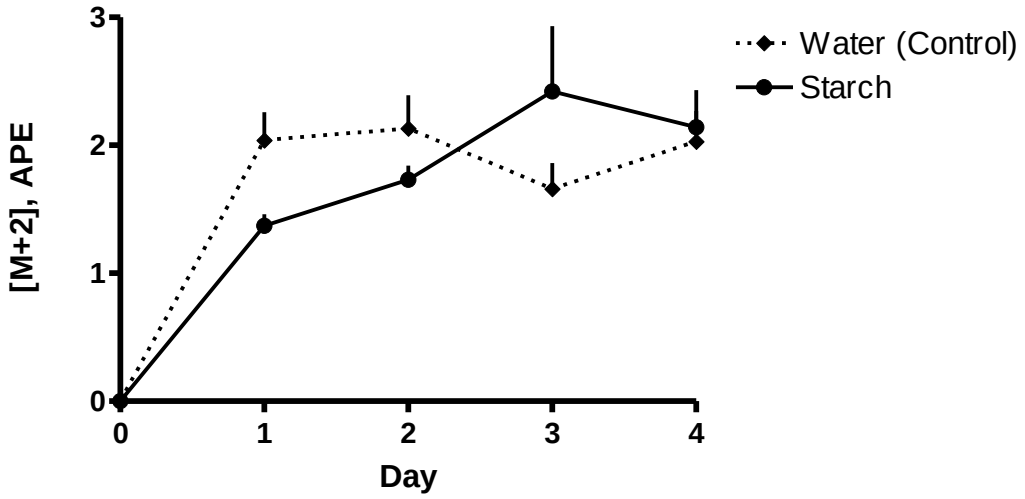


Figure 2.1: The time-course of urinary [$^{15}N_2$]urea enrichments. Values are the mean of 4 sheep (± standard error).

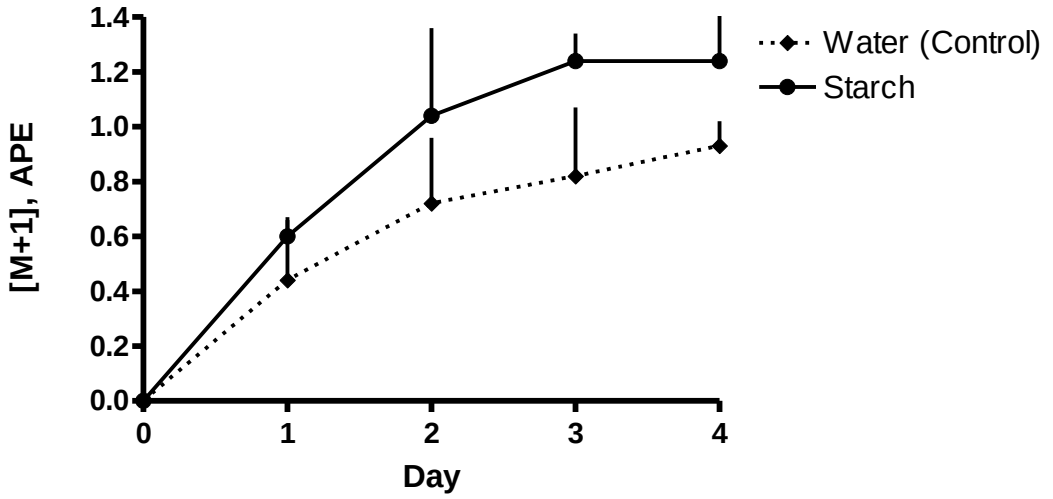


Figure 2.2: The time-course of urinary [$^{15}N^{14}N$]urea enrichments. Values are the mean of 4 sheep (± standard error).

Table 2.4: Urea-N fluxes in growing sheep (n=4) infused with starch or water (control).

	Water (control)	Starch	SE	*P*
Urea flux (g N/d)				
UER	16.3	17.6	1.51	NS
UUE	6.6	5.2	0.63	0.108
GER	9.7	12.3	1.86	NS
ROC	5.5	5.9	1.09	NS
UFE	1.0	1.9	0.22	0.047
UUA	3.2	4.5	1.47	NS
Fractional transfers				
UER to urine (u)	0.41	0.31	0.05	NS
GER to ROC (r)	0.53	0.50	0.07	NS
GER to feces (f)	0.10	0.17	0.03	NS
GER to UUA (a)	0.36	0.33	0.09	NS
Plasma urea conc. (mM)	4.01	3.02	0.71	NS

UER = urea entry rate; UUE = urinary urea elimination; GER = gut entry rate; ROC = return to ornithine cycle; UFE = urea-N fecal elimination; UUA = urea-N used for anabolism.

Glucose entry and gluconeogenesis: Glucose entry rate, gluconeogenesis and Cori cycling were all increased ($P < 0.05$) with starch infusion (**Table 2.5**). There was no difference in plasma glucose concentration.

Table 2.5: Glucose kinetics in growing sheep (n=4) infused with starch or water (control).

	Water (control)	Starch	SE	*P*
Glucose kinetics (g/d)				
Glucose entry rate	127.9	177.2	11.46	0.029
Cori recycling	29.5	45.4	2.24	0.035
Gluconeogenesis	98.4	131.9	9.96	0.033
Plasma glucose (mM)	3.8	4.1	0.26	0.200

Discussion

In this study, starch was infused into the rumen of sheep to recapitulate the changes in rumen VFA and effects on urea-N kinetics and gluconeogenesis that are characteristic of feeding a concentrate-type diet. By doing so, we aimed to establish relationships between rumen VFA profiles and N kinetics that would allow identification of specific VFA that might be primary regulators of urea-N recycling and gluconeogenesis. The basal diet supplied ~340 g starch per day, and the additional 100 g starch infused into the rumen was designed to shift rumen VFA profiles to more closely reflect the rumen fermentation conditions when feeding a high concentrate diet (i.e. 80% grain) where higher propionate and (or) butyrate concentrations are observed (Abdul–Razzaq and Bickerstaffe, 1989; Shen et al., 2004). Despite our attempts, there was no effect of starch infusion on total rumen VFA nor on the concentrations and molar proportions of VFA. Furthermore, there were occasional feed refusals of this diet in their first period that necessitated feeding the sheep in the second period at levels below the

target of 1.5× maintenance energy requirements in order to keep feed intakes equal in the two periods. In consequence, the sheep were not gaining weight at a rate close to their genetic potential, and this may have limited their responses to starch infusion. Nonetheless, there were several observations in this study that are noteworthy.

It has often been observed that feeding high-starch diets results in lower apparent N-digestibility (Orskov, 1986). In the present study, apparent N-digestibility was reduced (~5%) with ruminal starch infusion and there was an increase in fecal-N excretion that was largely due to the increase in urea-N excretion in feces. This equivalent magnitude of increase in UFE and fecal-N output with starch infusion suggests that a portion of the infused starch escaped the rumen to the cecum and large intestines where it was fermented by hindgut bacteria leading to an increase in urea-N partition to the hindgut. The greater UFE with starch infusion was offset by a reduction in UUE, thus there was no difference in N retention between treatments. Overall, with no change in urea entry, starch infusion shifted the elimination of urea-N from urine to feces. Furthermore, it is possible that the level of starch in the basal diet (35%) had maximized rumen microbial fermentation (or metabolism), and thus the infused starch was unable to further enhance fermentation and promote increased capture of N for microbial protein synthesis.

Despite no change in rumen VFA concentrations, the starch infusion did lead to an increased rumen synthesis and absorption of glucogenic substrates that resulted in increased glucose entry rate and gluconeogenesis. On an energetic basis, the increase in glucose entry (~50 g) accounted for ~50% of the starch energy (1.67 MJ) infused. Two-thirds of the increase in glucose entry could be accounted for by the increase in

gluconeogenesis (~34 g), suggesting that the majority of the infused starch was fermented in the rumen, most likely to propionate.

In summary, under the feeding conditions of this study, starch infusion into the rumen failed to achieve significant changes in rumen butyrate and propionate concentration. In consequence, there were no effects of rumen starch fermentation on urea-N recycling and N retention, despite enhanced gluconeogenesis. As a result, the study was unable to provide potential links between rumen VFA profiles and effects on urea-N recycling. It is probable that the effect of starch infusion was masked by the high level of starch in the basal diet, as there are studies which show a response to starch when a forage type diet is used (eg. Huntington et al., 2009). In conclusion, further experiments will be conducted to investigate the direct role of individual VFA by infusion into the rumen of propionate and butyrate and measurements of urea-N kinetics and N utilization in growing sheep.

CHAPTER 3: ROLE OF RUMEN BUTYRATE IN REGULATION OF NITROGEN UTILIZATION AND UREA NITROGEN KINETICS IN GROWING SHEEP

Abstract

Urea-N recycling is a key process that has the potential to improve protein and amino acid efficiency in ruminants. Butyrate, a major rumen volatile fatty acid (VFA), has been indirectly linked to enhancement of urea recycling based on higher expression of urea transporter (UT-B) in the rumen epithelia of steers fed a rumen butyrate-enhancing diet. The aim of these studies was to determine the possible role of rumen butyrate on urea-N recycling by direct infusion of butyrate into the rumen of growing sheep. Two studies were conducted to quantify the effect of enhanced rumen butyrate concentrations on N balance, urea kinetics and rumen epithelial proliferation. Wether sheep (n=4), fitted with a rumen cannula, were fed to 1.8 × maintenance energy intake a pelleted ration (130 g CP/kg, 9.3 MJ ME/kg) and infused into the rumen with either a control solution or butyrate (mimicking a concentrate diet) for 9-d periods in a balanced crossover design. In Exp 1, sheep were given intra-ruminal infusions of either a electrolyte buffer solution (Con-Buf) or butyrate dissolved in the buffer solution (But-Buf). In Exp 2, sheep were given intra-rumianl infusions of either sodium-acetate (Na-Ac) to simulate rumen conditions of a forage-based diet or infusion of sodium butyrate (Na-But). All solutions were pH adjusted and VFA were infused at 10% of ME intake. [$^{15}N_2$]urea was continuously infused i.v. for the last 5 d of each period, and total urine

collected by vacuum and feces by a harness bag. In Exp 1, [ring-D_5]phenylalanine was continuously infused i.v. over the last 12 h, after which a biopsy from the rumen papillae was taken for measurement of fractional protien synthesis rate (FSR). Butyrate infusion treatments increased ($P = 0.1$ in Exp 1; $P < 0.05$ in Exp 2) the proportion of rumen butyrate, and acetate infusion increased ($P < 0.05$) rumen acetate. All animals were in positive N balance (4.2 g N/d in Exp 1; 7.0 g N/d in Exp 2), but no difference in N retention was observed between treatments. In Exp 2, urea entry (synthesis) rate was reduced ($P < 0.05$) by Na-But compared to the Na-Ac control. As a result, a higher proportion of urea-N entering the rumen was utilized for microbial protein synthesis in Exp 2 with Na-But infusion. In Exp 1, although But-Buf infusion increased the FSR of rumen papillae (35.3 ± 1.08 %/d vs 28.7 ± 1.08 %/d, $P < 0.05$), urea kinetics were not altered by But-Buf compared to Con-Buf. This study is the first to directly assess the role of butyrate in urea recycling and its effects on rumen papillae protein turnover in growing lambs. Under the feeding conditions and the continuous rate of butyrate infusion into the rumen in the present studies, butyrate does not affect overall N retention in growing sheep. However, butyrate may play a role in regulating urea synthesis and thus affect the redistribution of urea-N fluxes in the overall scheme of N metabolism.

Introduction

Nitrogen (N) metabolism and use in ruminants is a complex process. Almost 50-100% of the N digested by ruminant animals passes through the urea-N pool (Lapierre and Lobley, 2001), and hence is subject to the fates of urea including excretion in urine or transfer and subsequent utilization in the gastro-intestinal tract (GIT) by bacteria. It has long been established that rumen fermentation, affected by the diet, can influence the

partitioning and use of urea-N in ruminants. For example, feeding a highly fermentable concentrate diet increases the amount of urea-N recycled to the rumen (Kennedy and Milligan, 1980; Huntington, 1989) and increases the capture of rumen ammonia (NH_3) by microbes (Stern and Hoover, 1979) leading to lower urinary N losses and hence, greater N retention (Fluharty et al., 1999). However, the mechanisms that dictate urea-N kinetics are still not well understood. Rumen volatile fatty acids (VFA) are a primary product of rumen fermentation, and are responsible for meeting ~70-80% of the animals energy needs (Bergman and Wolff, 1971). Among the VFA, butyrate, which makes up about 10-20% of the total rumen VFA (Bergman, 1990), may play a key role in regulation of urea-N kinetics and use as suggested by recent studies. Higher rumen butyrate which results from feeding of highly fermentable diets (Shen et al., 2004), may affect urea-N kinetics through a wide range of mechanisms either directly by rumen proliferation (Sakata and Tamate, 1978) or indirectly by enhancing rumen tissue blood flow, permeability and absorptive capacity of the rumen (Storm et al., 2011). In steers fed a concentrate diet, higher ruminal urea transporter (UT-B) expression was associated with higher rumen butyrate levels (Simmons et al., 2009). Increased expression of UT-B by the rumen epithelia might directly affect the entry of plasma urea into the rumen. On the other hand, butyrate may indirectly enhance gut entry of plasma urea by increasing the blood flow to rumen epithelia (Storm et al., 2011) or by promoting keratinization of rumen epithelia, hence increasing its permeability (Norton et al., 1982). There is mounting evidence demonstrating that butyrate promotes rumen epithelial development, however, most of the measurements are morphological (increased papillae length and surface area) or histological (mitotic indices) in nature (Sakata and Tamate, 1978; Simmons et al., 2009).

Lastly, higher rumen butyrate may inhibit the use of propionate by the liver (Krehbiel et al., 1992) and substitute the use of glucose by rumen epithelia (Baldwin and Jesse, 1992). These glucose and propionate sparing effects may avoid additional amino acid (AA) catabolism for energy production. Therefore, it also remains unclear whether the effect of butyrate is driven by energy or if it is an effect unique and directly attributable to butyrate.

To answer these questions about the role of rumen butyrate in N utilization and urea-N kinetics, we conducted two experiments. In the first experiment, rumen butyrate was artificially elevated by constant infusion into the rumen compared with buffer as control. In the second experiment, iso-energetic infusions of acetate compared to butyrate were used to simulate high forage compared to high concentrate fed rumen fermentation profiles, feeding conditions where urea-N recycling differences have been observed.

Materials and methods

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland (protocol number R-10-45).

Animals, diets and treatment periods

Experiment 1: Upon arrival in the ANSC animal facilities, Polypay × Dorsett wether lambs (28.5 ± 3.49 kg BW) were transitioned to a standard pelleted diet (**Table 3.1**) and fed to 1.8 × maintenance energy requirements. After acclimation and a 30-d quarantine period, sheep were surgically fitted with a rumen cannula (Appendix 1). Following recovery from surgery, sheep were placed into individual metabolic crates and fed an

equal amount of the basal diet every 2 h via an automatic feeder. Four cannulated sheep were assigned to receive continuous (1 L/d) rumen infusions of a buffer (Con-Buf) and buffered butyrate (But-Buf) in a balanced cross-over design with each infusion period lasting 8 d (**Table 3.2**). The butyrate infusion rate was calculated to supply 10% of ME (1 MJ/d; based on gross energy of butyrate) and to increase rumen butyrate concentration by ~40% as was observed by Simmons et al. (2009). The infusates was adjusted to pH 6.8 and electrolyte load was equal in both solutions. Each infusion period was separated by 3 wk during which sheep were placed into individual floor pens for exercise and to allow a period of treatment washout.

Experiment 2: The same Polypay × Dorsett wether sheep (42 ± 6.6 kg BW) from Exp 1 were used in this experiment. In this experiment, four sheep received iso-energetic (1 MJ/d, 10% of ME intake based on gross energy of acetate and butyrate) and isonatremic intra-ruminal infusions of sodium acetate (control, Na-Ac) and sodium butyrate (Na-But) in a balanced cross-over design with each infusion period lasting 9 d (**Table 3.2**). The levels of VFA infused were designed to simulate high forage (Na-Ac) compared to high concentrate (Na-But) fed rumen fermentation profiles, feeding conditions where urea-N recycling differences have been observed. The solutions were adjusted to pH 8 and NaOH and Na-formate were used to balance the Na-But treatment to the same level of sodium as in the Na-Ac treatment. Each period was separated by 5 d during which sheep were placed in individual floor pens for exercise and washout.

Table 3.1: Composition of diet for Exp 1 and Exp 2.

Item	Amount, g/kg of diet (DM basis)	
Ground Corn	385	
Alfalfa	540	
Soybean Meal	50	
Vitamin-mineral premix[1]	25	
Nutrient composition (by chemical analysis)		
	Exp 1	**Exp 2**
DM	902	877
CP	166	165
Soluble Protein	47	41
ADF	187	164
NDF	246	226
Starch	304	351
Crude fat	33	33
TDN	702	730
Nem, MJ/Kg	6.83	7.19
Neg, MJ/Kg	4.34	4.61

[1]Bel Air sheep mineral vitamin mixture (per kg of premix): Ca, 210 g; P, 30 g; NaCl, 160 g; S, 30 g; Mg, 25 g; K, 24 g; Fe, 2.4 g; Mn, 2.4 g; Zn, 2.7 g; Se, 24 mg; Co, 30 mg; I, 40 mg; Choline-Cl, 4.41 g; vitamin A, 661,500 IU; vitamin D_3 132,300 IU; vitamin E, 1,764 IU.

DM: dry matter; CP: crude protein; ADF: acid detergent fiber; NDF: neutral detergent fiber; TDN: total digestible nutrients

Table 3.2: Composition of treatment infusion solutions for Exp 1 and Exp 2.

	Exp 1		Exp 2	
	Buffer (control)	**Butyrate + Buffer**	**Acetate (control)**	**Butyrate**
Infusate (g/L)				
Sodium acetate, trihydrate			242	
Sodium butyrate				71.3
Sodium formate				76.9
Sodium bicarbonate	18.3	18.3		
Potassium bicarbonate	9.5	9.5		
Sodium chloride	1.8	1.8		
Butyric acid		40		

Isotope infusion

Temporary jugular vein catheters were inserted at least 2 d prior to initiating isotope infusions. Over the last 4 (Exp 1) or 5 (Exp 2) days of each treatment period a sterile solution containing [$^{15}N_2$] urea (99 atom percent ^{15}N, Cambridge Isotope Laboratories Inc., Andover, MA) was continuously infused (0.6 g/d) through a jugular vein catheter. It has been shown that this length of [$^{15}N_2$]urea infusion results in the attainment of isotopic plateau in urinary [$^{15}N^{14}N$] and [$^{15}N^{15}N$]urea enrichment and that this infusion rate increases the urinary [$^{15}N^{15}N$]urea enrichment to 1.5-2.5 atoms percent excess (APE) (Sunny et al., 2007).

The fractional rate of rumen epithelial protein synthesis (FSR) was determined in Exp 1. During the last 8 h, D_5 phenylalanine tracer (170 mg D_5-phenylalanine in 125 g saline) was continuously infused i.v. along with urea tracer through jugular catheter after priming with a bolus dose (42.5mg D_5-phenylalanine in 3 g saline). This amount of tracer was calculated and tested in a pilot trial in order to achieve a 4% enrichment of rumen epithelia. Exact starting and ending infusion times were carefully recorded.

Sampling

During the last 5 d of each treatment period, sheep were fitted with a harness for total collection of feces (by bag) and urine (by suction), which were weighed and recorded. A slight vacuum was used to collect urine directly into a sealed container placed on a stir plate and containing sufficient HCl to reduce urine pH to < 3 to prevent bacterial hydrolysis of urea and ammonia volatilization. Well-mixed sub-samples from each collection (100 g feces and 40 g urine) were stored at -20^{o}C for later analysis. Over

the last 6 h of tracer infusion, urine samples were also collected at 2 h intervals and later analyzed to verify that plateau of [$^{15}N_2$] urea enrichment in urine had been attained. Hourly blood samples were collected during the last 5 h. Plasma was separated by centrifugation (8,000 × *g* for 15 min at 4^{o}C) and stored at -20^{o}C for later analysis. At the end of each infusion period, following collection of the last sample, sheep were removed from crates and within 10 mins the rumen was completely evacuated by vacuum. A rumen papillae biopsy sample was obtained after the rumen was locally rinsed with saline (Apendix 2). The biopsy samples were stored at -20^{o}C. The rumen fluid was well mixed and a representative sample (50 mL) strained through two layers of cheese cloth. The strained rumen fluid was immediately centrifuged (8,000 × *g* for 10 min at 4^{o}C) and the pH of the supernatant recorded using a pH indicator strip (VWR International, West Chester, PA). For determination of VFA concentration, to a known amount (1 g) of rumen fluid was added 0.25 g of an internal standard (260 mM 1-^{13}C-Na-acetate, 80 mM methyl-D_3-propionic acid and 40 mM 1-^{13}C-Na-butyrate), and the samples stored at -20^{o} C for later analysis.

Urea concentration and enrichment

The concentration and enrichment of plasma and urinary urea were determined by Gas Chromatography-Mass Spectrometry (GC-MS, Agilent 6890 series gas chromatography system coupled to an Agilent 5973N mass selective detector; Agilent; Palo Alto, CA) under electron ionization (EI) mode. For determination of urinary urea concentration, to a known weight (0.25 g) of an internal standard solution containing [^{13}C, $^{15}N_2$] urea (5 mg/g) was added to an equal amount of urine. For plasma urea concentration, a known amount (500 mg) of internal standard containing [^{13}C, $^{15}N_2$] urea

(0.26 mg/g) was added to an equal amount of plasma. Plasma and urine samples were acidified by adding an equal volume of 15% sulfosalicylic acid and 10% trichloroacetic acid (w/v) respectively and centrifuged for 10 min at 10,000 × *g* (room temperature) to precipitate proteins and debris. Urea was isolated from acidified samples by application to 0.5 g of cation exchange resin (AG 50W-X8 resin, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA). The resin was washed with 2 x 2 mL of water and urea eluted with 2 mL of ammonium hydroxide plus 1 mL of double distilled water. An aliquot (100 to 200 µl) of the eluate was dried under N_2 gas and tertiary-butyldimethylsilyl derivatives of urea were prepared by adding 50 µl each of acetonitrile (Pierce chemicals , Rockford, IL) and N-methyl-N-t-butyl-dimethylsilyl-trifluroacetamide (Pierce chemicals , Rockford, IL) followed by heating at 90^{o}C for 20 minutes (El-Kadi et al., 2006). Derivatized urea samples were separated on a fused silica capillary column (HP-50; 30 m × 0.25 mm × 1 µm Hewlett-Packard, Palo Alto, CA) prior to MS under EI conditions. Ions (mass-to-charge; m/z) 231.2 (unlabeled; M), 232.2 (singly labeled; M+1), 233.2 (doubly labeled; M+2), and 234.2 (internal standard; M+3) were monitored using selected ion monitoring (SIM), with conditions of: an initial temperature of 150^{o}C followed by 15^{o}C/min to 250^{o}C. Urea concentration in the samples was calculated by isotope dilution (Calder et al., 1999) based on the ratios of M+3/ M after correction for background (natural abundance) and spillover effects of M+2 urea.

Total urinary and fecal N and fecal ^{15}N

Total urinary and fecal N was measured using an automated N analyzer (CN-2000, Leco, St. Joseph, MI). Fecal samples were dried in a forced air drying oven at 60^{o}C for 5 d. Dried samples were pulverized in a liquid nitrogen freezer mill (Freezer-Mill

6850, Spex CertiPrep Inc., Metuchen, NJ) and samples submitted to the stable isotope facility at the University of California-Davis (CA) for ^{15}N analysis by isotope-ratio mass spectrometry.

Rumen fluid VFA concentration

Samples were thawed and acidified by adding 250 µL of 2 M HCl, and centrifuged for 15 min at 13,000 × *g* at room temperature to precipitate solids. The supernatant was filtered through a 0.2 µm filter (Nalgene, Rochester, NY), and anhydrous ether (0.5 mL) added to extract VFA. An aliquot (200 µl) of extracted VFA was converted to the tert-butyldimethylsilyl derivative by adding 50 µL MTBSTFA followed by heating at 80^{o}C for 20 min (Duncan et al., 2004). After samples had cooled, 50 µL of methanol was added to react with the surplus MTBSTFA. Derivatized samples were injected onto the GC column (fused silica capillary column, HP-5; 30 m × 0.25 mm × 1 µm Hewlett-Packard) with the GC inlet set at 250°C and the following column conditions: initial temperature of 60^{o}C held for 3 min followed by 10^{o}C/min to 210^{o}C for 4 min. Ions of m/z 117 and 118 for acetate, 131 and 134 for propionate and 145 and 146 for butyrate were monitored. VFA concentrations were calculated by isotope dilution (Calder et al., 1999).

Phenylalanine enrichment in plasma and rumen papillae

Plasma samples were acidified using 15% sulfosalicylic acid and centrifuged for 10 min at 10,000 × *g* at room temperature to precipitate proteins. The supernatant was desalted by ion-exchange by application to 0.5 g of cation exchange resin (AG 50W-X8 resin, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA). The resin was washed with 2

x 2 mL of water and AA fraction eluted with 2 mL of ammonium hydroxide plus 1 mL of double distilled water. The eluate was lyophilized to dryness. AA were converted to tertiary-butyldimethylsilyl derivatives by adding 50 µl each of acetonitrile (Pierce chemicals , Rockford, IL) and MTBSTFA followed by heating at 90^{o}C for 20 minutes (El-Kadi et al., 2006). For determination of rumen protein-bound phenylalanine enrichments, approximately 15 mg of wet tissue was deproteinized and homogenized by washing with 15% ice-cold sulfosalicylic acid. Homogenized tissue samples were hydrolyzed by adding 4 mL of 2 M HCl and heating at 110^{o}C for 18 h. The acid hydrolysate was desalted by ion-exchange and derivatized to obtain tertiary-butyldimethylsilyl derivatives similar to plasma samples. Derivatized AA samples were separated on a fused silica capillary column (HP-50; 30 m × 0.25 mm × 1 µm Hewlett-Packard, Palo Alto, CA) prior to MS under EI conditions. Ions (mass-to-charge; m/z) 336 (unlabeled; M) and 341 ([D_5]phenylalanine; M+3) were monitored using SIM, with conditions of: an initial temperature of 150^{o}C followed by 15^{o}C/min to 250^{o}C. Phenylalanine enrichments were expressed as APE relative to background natural abundance. The obtained data was corrected for deuterium-exchange using a calibration curve.

Calculations

Urea-N Kinetics: Urea-N kinetic calculations were based on Lobley et al. (2000). Whole body urea synthesis (urea entry rate; UER) was estimated from the dilution of the infused [$^{15}N_2$]urea tracer. A portion of UER is excreted into urine (UUE), whereas the remainder enters the gut tissues (GER). Urea entering the gut has three different fates: 1) excretion in feces (UFE), 2) hydrolysis by rumen microbes with absorption of NH_3 and

return to the ornithine-urea cycle (ROC), and 3) utilization by gut microbes for protein synthesis (anabolic use, UUA). UFE and ROC are estimated directly from excretion of fecal ^{15}N and based on the appearance of urinary [$^{15}N^{14}N$]urea, respectively. UUA is estimated by the difference between GER and UFE+ROC. An important assumption in this model is that all the doubly labeled urea in urine is derived from the infused tracer. Calculations for urea-N kinetics are shown in Appendix 3.

FSR and whole body protein flux: Due to lack of sufficient free pool phenylalanine in rumen papillae biopsies, plasma enrichments were used in the calculation. FSR was calculated from the average enrichment of plasma and rumen protein-bound D_5 phenylalanine using the following formula (Connell et al., 1997):

$$\text{FSR (\% d}^{-1}) = \frac{\text{Rumen Protein bound Phe Enrichment (APE)} \times 100}{[\text{Average plasma Phe Enrichment (APE)} \times \text{Infusion time (d)}]}$$

Whole body protein flux was calculated from the irreversible-loss rate (ILR, flux) of phenylalanine using following calculations (Savary-Auzeloux et al., 2003).

$$\text{ILR (g d}^{-1}) = \left[\frac{\text{Tracer purity(\%)}}{\text{Average plasma Phe Enrichment (APE)}} - 1\right] \times \text{Infusion rate (g d}^{-1})$$

and,

$$\text{Whole body protein flux (g d}^{-1}) = \text{ILR (g d}^{-1}) \times 29.33$$

The factor 29.33 is obtained based on phenylalanine content of total body protein in sheep (Savary-Auzeloux et al., 2003).

Statistical Analysis

Animals were nested within groups. Treatment and period were considered to be fixed effects and animal within group were considered to be random effects. Data were analyzed using the PROC MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). The following linear model was used to analyze the data:

$$Y_{ijk} = \mu + group_i + animal_{ij} + period_k + treatment_h + error_{ijk}$$

where,

Y_{ijk} = response variable (UER, UUE etc.) in the k^{th} period of the j^{th} animal in the i^{th} group (i = 1, 2; j = 1 to n; k = 1, 2)

μ = the overall mean effect

$group_i$ = the effect of the i^{th} group (i = 1,2)

$animal_{ij}$ = the effect of the j^{th} animal on the i^{th} group (j = 1, 2, .. , n_i)

$period_k$ = the effect of the k^{th} period (k = 1,2)

$treatment_h$ = the effect of the h^{th} treatment (h = 1,2; being a function of i & k)

$error_{ijk}$ = the residual error

For FSR measurements, data for only 3 animals was used as one of the animals had a longer washout period. Statistical significance was set at $P \leq 0.05$ and a trend at $P \leq 0.10$.

Results

Experiment 1: Butyrate vs Buffer (control)

Feed intake, digestibility and N-retention: All sheep gained weight during the experiment. Dietary DM (67 to 73%) and N digestibility (70 to 76%) did not differ between treatments. (**Table 3.3**). There was no effect of But-Buf on N retention, however, urinary N excretion tended (P = 0.104) to increase (~1 g urea-N/d) by But-Buf, which accounted for most the difference in urinary urea output. No difference was observed in fecal N output.

Rumen pH and VFA profiles: The rumen pH was not altered by treatments (**Table 3.4**). The molar proportion (mol/100 mol VFA) of rumen butyrate numerically increased ($P = 0.107$) with But-Buf infusion, although the absolute concentration of butyrate did not change (**Table 3.4**). Rumen acetate proportion tended ($P = 0.065$) to decrease with infusion of But-Buf, but its concentration was not different between treatments. Surprisingly, the concentration of rumen propionate was elevated ($P < 0.05$) with But-Buf infusion, but there was no difference in the proportion of propionate between treatments.

Urea-N kinetics: For both treatments, urinary [M+2] urea enrichment reached an isotopic plateau (**Figure 3.1**) within 2 d of [$^{15}N_2$] urea infusion. Urinary [M+1] urea enrichment increased until attaining a plateau between 2 and 3 d of infusion (**Figure 3.2**). Total fecal ^{15}N enrichment continued to increase throughout the infusion period, attaining a final value approximately 80% of predicted plateau. Hence, the fecal ^{15}N enrichment on the last day was used in calculations. Urea-N kinetics are shown in **Table**

3.5. Infusion of But-Buf tended ($P = 0.082$) to increase UER. When considered on a digestible N basis, UER was significantly increased ($P < 0.05$) by ~8% with But-Buf. The absolute amounts of GER and ROC tended ($P \leq 0.1$) to increase by the same amount of ~1 g urea-N/d. Fractional transfers to urine and feces were not affected by But-Buf infusion, whereas the proportion of urea-N returned from GIT (ROC:GER) increased ($P < 0.05$).

FSR of rumen epithelia and whole body protein flux: Fractional protein synthesis of rumen epithelia was increased ($P = 0.05$) by But-Buf (35.3 ± 1.08%/d) compared to Con-Buf (28.7 ± 1.08%/d). These results, however, were obtained from the data of only three sheep, i.e. one sheep was dropped from this analysis because of a large gap between experimental periods. Whole body protein flux did not differ between But-Buf (185 ± 9.2 g/d) and Con-Buf (196 ± 9.2 g/d).

Experiment 2: Butyrate vs Acetate (control)

Feed intake, digestibility and N balances: All animals gained weight during the experiment. Dietary DM (60 to 72%) and N (60 to 70%) digestibility did not differ between treatments (**Table 3.3**). Total urinary N excretion tended ($P = 0.091$) to be lower with Na-But compared to Na-Ac, whereas there were no differences in fecal N output and N retention. Similar to Exp 1, the difference in total urinary N output was largely accounted for by the difference in urinary urea-N.

Table 3.3: Nitrogen balance in growing sheep (n=4) in Exp 1 and Exp 2.

	Exp 1				Exp 2			
	Buffer (control)	**Butyrate + Buffer**	**SE**	***P***	**Acetate (control)**	**Butyrate**	**SE**	***P***
N balances[1] (g N/d)								
N intake	23.0	23.3	0.83	NS	27.8	26.9	2.60	NS
N in urine	12.4	13.5	0.37	0.104	11.6	10.4	0.73	0.091
N in feces	6.2	5.8	0.39	NS	9.4	9.5	1.40	NS
N retained	4.4	4.0	0.65	NS	6.8	7.0	0.73	NS
DM digestibility (%)	70.9	72.0	0.93	NS	67.9	66.2	2.01	NS
N digestibility (%)	72.9	75.2	1.36	NS	66.7	65.1	1.86	NS
Urea-N:urine-N	0.79	0.81	0.03	NS	0.80	0.74	0.03	NS
UER:digestible N	0.92	1.0	0.05	0.039	0.95	0.84	0.02	0.017
N retained:N digested	0.26	0.23	0.03	NS	0.39	0.40	0.07	NS

[1]Nitrogen balance was measured during the last 4 d of each 9 d treatment period.

Table 3.4: Rumen volatile fatty acid profiles in growing sheep (n=4) in Exp 1 and Exp 2.

	Exp 1				Exp 2			
	Buffer (control)	**Butyrate + Buffer**	**SE**	***P***	**Acetate (control)**	**Butyrate**	**SE**	***P***
Concentration (mM)								
Acetate	129.9	135.3	17.89	NS	168.3	131.7	14.57	NS
Propionate	14.9	23.9	1.28	0.019	23.7	22.1	3.00	NS
Butyrate	11.4	24.3	7.14	NS	12.1	20.8	1.42	0.012
Molar proportion (mol/100 mol)[1]								
Acetate	83.1	74.0	2.22	0.065	82.5	75.5	0.57	0.006
Propionate	9.7	13.2	1.10	NS	11.5	12.5	0.65	NS
Butyrate	7.2	12.8	2.42	0.107	6.0	12.0	0.64	0.022
pH	6.4	6.4	0.17	NS	6.4	6.8	0.15	NS

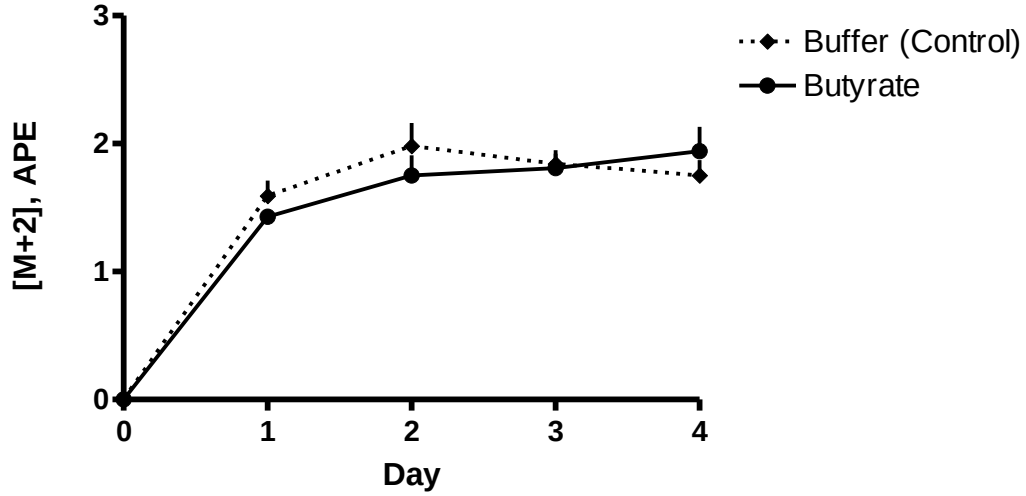
[1] Moles of each VFA relative to the sum of acetate, propionate and butyrate.

Table 3.5: Urea-N fluxes in growing sheep (n=4) in Exp 1 and Exp 2.

	Exp 1				Exp 2			
	Buffer (control)	**Butyrate + Buffer**	**SE**	***P***	**Acetate (control)**	**Butyrate**	**SE**	***P***
Urea flux (g N/d)								
UER	15.4	17.6	0.57	0.082	17.5	14.5	1.23	0.031
UUE	9.7	10.9	0.54	NS	9.3	7.8	0.88	0.105
GER	5.6	6.7	0.36	0.082	8.2	6.8	0.64	NS
ROC	3.5	4.6	0.39	0.102	5.8	3.7	0.42	0.024
UFE	1.0	0.8	0.17	NS	1.8	1.6	0.21	NS
UUA	1.1	1.3	0.36	NS	0.6	1.5	0.42	NS
Fractional transfers								
UER to urine (u)	0.64	0.63	0.025	NS	0.53	0.53	0.029	NS
GER to ROC (r)	0.63	0.72	0.056	0.026	0.70	0.56	0.051	NS
GER to feces (f)	0.18	0.12	0.028	NS	0.22	0.24	0.018	NS
GER to UUA (a)	0.20	0.17	0.068	NS	0.08	0.20	0.065	NS
Plasma urea conc. (mM)	3.76	3.97	0.632	NS	3.32	3.39	0.333	NS

UER = urea entry rate; UUE = urinary urea elimination; GER = gut entry rate; ROC = return to ornithine cycle; UFE = urea-N fecal elimination ; UUA = urea-N used for anabolism.

A)



B)

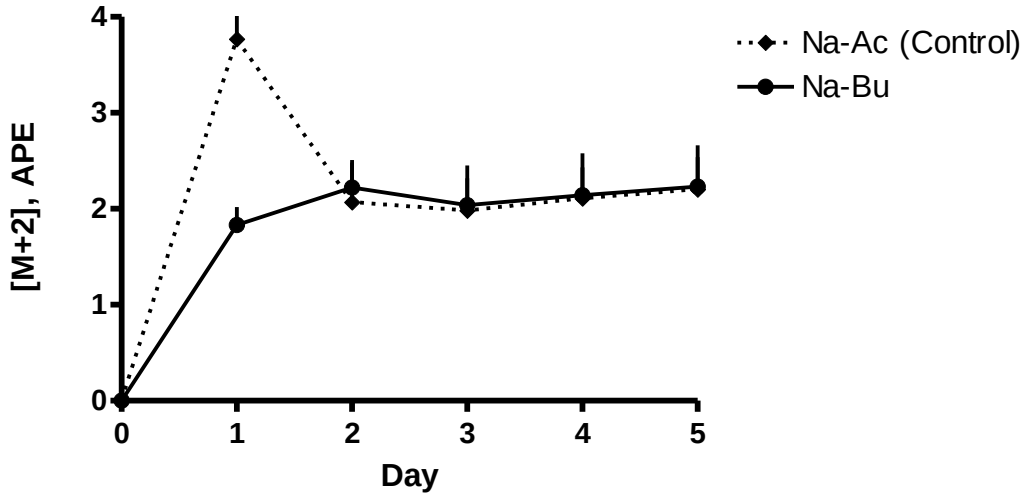
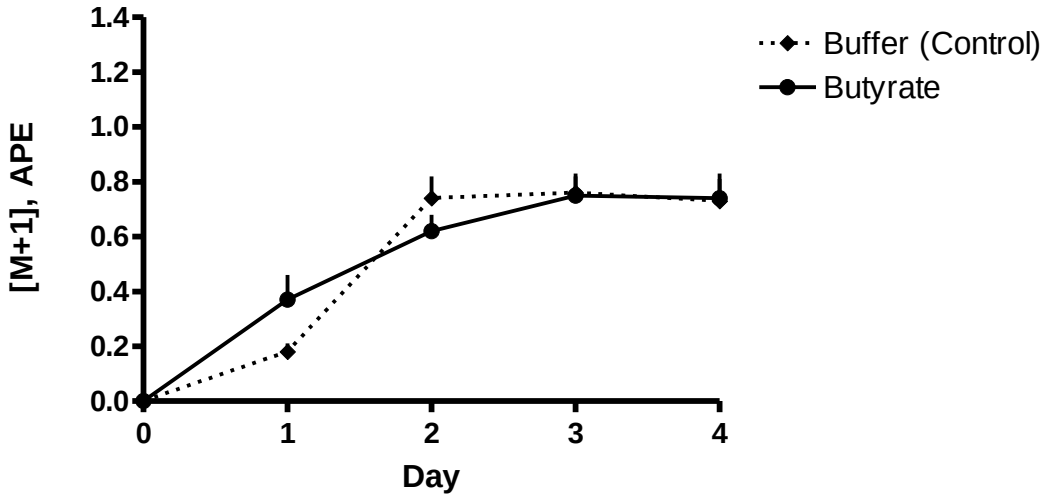


Figure 3.1: The time-course of urinary [$^{15}N^{15}N$]urea enrichments in Exp 1 (A, 4 day [$^{15}N_2$]urea infusion) and Exp 2 (B, 5 day [$^{15}N_2$]urea infusion). Values are the mean (± standard error).

A)



B)

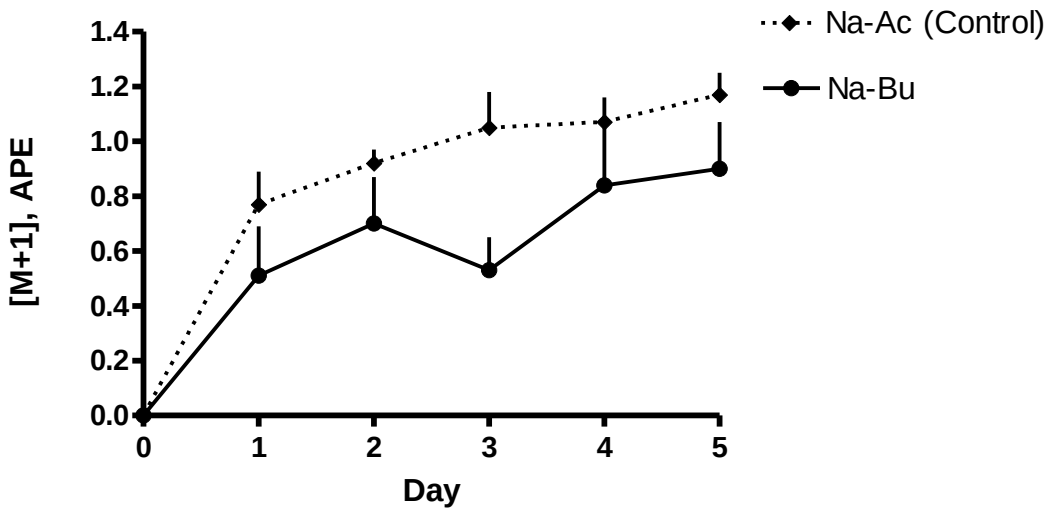


Figure 3.2: The time-course of urinary [$^{15}N^{14}N$]urea enrichments in Exp 1 (A, 4 day [$^{15}N_2$]urea infusion) and Exp 2 (B, 5 day [$^{15}N_2$]urea infusion). Values are the mean (± standard error).

Rumen pH and VFA profiles: Rumen pH did not differ between treatments. The molar proportion (mol/100 mol VFA) and concentration of rumen butyrate were higher ($P < 0.05$) with Na-But (**Table 3.4**) whereas the concentration of rumen acetate was higher ($P < 0.05$) with Na-Ac infusion.

Urea-N kinetics: For all treatments, [M+2] urea enrichment in urine reached isotopic plateau (**Figure 3.1**) within 2 d of [$^{15}N_2$] urea infusion. Urinary [M+1] urea enrichment continued to increase until attaining a plateau after 2 to 3 d of infusion (**Figure 3.2**). Total fecal ^{15}N enrichment continued to increase throughout the infusion period, hence values on the last day (over 72-96 h) were used in calculations. Urea-N kinetics are shown in **Table 3.5**. UER was higher ($P < 0.05$) for Na-Ac compared to Na-But as well as when considered on a digestible N basis (UER:digestible N). Urinary urea-N excretion tended ($P = 0.105$) to be lower with Na-But infusion and ROC was lower ($P < 0.05$) compared to Na-Ac. Fractional transfers of urea-N were not different between treatments.

Discussion

Among the major rumen VFA, butyrate has been suggested as a likely candidate responsible for enhancing urea-N entry and degradation in the rumen (Norton et al., 1982**)**. Simmons et al. (2009) observed higher expression of urea transporter (UT-B) in the rumen epithelia of steers fed a high concentrate diet. Under their feeding conditions, rumen butyrate was elevated, and thus the authors hypothesized that the higher butyrate concentrations had increased rumen UT-B expression. However, when diets are fed that contain large amounts of fermentable material, there are dynamic changes in not just

rumen VFA but also rumen NH_3 concentrations and the rumen microbiota. Thus, it becomes difficult to attribute the observed effects (i.e., N balance or urea-N kinetics) to particular VFA, metabolites or other conditions in the rumen (e.g., microbial protein supply, ketone bodies, pH). A more direct approach to determine the relationship between rumen VFA and the mechanisms affecting urea-N recycling and N utilization in the rumen is by artificially manipulating the rumen environment by infusion of individual or mixtures of VFA. Thus, the aim of these two studies was to directly quantify the effects of altering rumen butyrate concentrations and supply on N balance and urea-N kinetics by direct infusion of butyrate into the rumen of growing sheep.

The rate of butyrate infusion in both studies was designed to supply an additional 10% of ME intake as butyrate to shift the rumen VFA profile towards that of a high concentration diet where butyrate concentrations are higher and under similar conditions as in the study by Simmons et al. (2009). The CP content of the diet was set at a level that supports moderate body weight gain (~200 g/d; NRC 2007) for sheep of the size used herein. All animals in the current study gained body weight during each treatment period, and all were in positive N balance (4.18 g N/d in Exp 1; 7.02 g N/d in Exp 2). Although there was a trend for butyrate infusions to alter urinary N output and urea-N fluxes, whole body N retention was not improved compared to infusion of Con-Buf or Na-Ac despite the potential for greater N retention by these growing sheep. Further, butyrate infusion did not affect either DM digestibility nor N digestibility.

There are many potential factors that can be altered when infusing VFA, such as rumen pH, osmotic (sodium) load and total energy infused, and thus we made every attempt to keep these variables constant between treatments. Butyrate infusions were

compared to buffer (Exp 1) and iso-energetic Na-Ac (Exp 2) infusions to directly assess effects of butyrate under conditions that simulate a high concentrate diet and a high forage diet, respectively. In both experiments, rumen VFA profiles were successfully altered as desired wherein butyrate infusion nearly doubled the proportion of rumen butyrate. A notable exception was in Exp 1 (buffer control) where, in addition to elevating rumen butyrate, rumen propionate was also elevated at the cost of acetate. Microbial fermentation is sensitive to rumen conditions. Recently, changes in rumen microbial populations were characterized in response to rumen butyrate infusion (Li et al., 2012), and where continuous infusion of butyrate (compared to buffer) enhanced rumen populations of *Succinimonas* bacterial species which primarily break down starch. Thus, the higher propionate observed on infusion of But-Buf may have shifted rumen microbial population to favor the fermentation of starch to propionate.

Liver production of urea was in the normal range expected for the N intakes supplied by the diet in the present studies (Huntington and Archibeque, 1999), however, confounding effects were found in the two experiments. In Exp 1 (buffer control), But-Buf tended to increase the UER by ~2.2 g urea-N/d. Of this increase in UER, half was recycled to the GI tract, but an equal amount was returned to the hepatic ornithine urea cycle. The diet used in the current studies contained ~30-35% starch (DM basis), and thus it is likely that the capture of N in the rumen had been maximized. Furthermore, while half of the increase in ureagenesis is due to increased ROC, the remainder is likely due to increased amino acid catabolism in the liver (Lobley et al., 1995). Indeed, ruminal butyrate infusion leads to an increase in glucagon secretion to promote hepatic gluconeogenesis (Obara et al., 1971), with amino acids serving as the primary substrates.

In Exp 2 (acetate control), there was ~3 g urea-N/d reduction in UER when Na-But was infused, and about ~2 g urea-N/d reduction in ROC. Lower UER tended to decrease urinary urea by ~1.5 g urea-N/d ultimately resulting in a numerically lower total urine N. Indeed, higher proportion of rumen acetate leads to a higher excretion of N in urine (Ørskov et al., 1991).

Modulation of urea-N fluxes across the GIT may indicate a higher permeability of the rumen wall brought about by butyrate infusion. Recently Storm et al. (2011) demonstrated a higher rumen epithelial blood flow resulting from butyrate infusion into emptied and washed rumen of cows, which also led to a higher permeability and greater absorption of nutrients across rumen wall. This may explain the tendency of higher gut entry of urea observed with But-Buf compared to Con-Buf herein.

It has been proposed that butyrate supplementation in milk replacer of early weaned calves may enhance development and growth of the rumen (Górka et al., 2011). The role of butyrate in proliferation of rumen epithelial cells has long been known (Sakata and Tamate, 1978). Simmons et al., (2009) found an increased gene expression of UT-B and higher rumen papillae length in concentrate fed steers. However, most measurements have been made using histological or morphological techniques. Our study is the first to establish that rumen butyrate infusion also increases the FSR of rumen epithelia, which is consistent with previous observations that butyrate enhances rumen papillae growth.

Our objective was to investigate the direct effect of elevating ruminal butyrate on urea-N recycling, overall N utilization and rumen tissue protein turnover rate, and thus to

provide direct evidence of a role for butyrate in upregulation of urea-N transfer to the rumen. Based on the results of these experiments, butyrate was not found to improve overall N retention in sheep, even though butyrate did alter urea synthesis and the redistribution of urea-N fluxes. The results are in agreement with Norton et al. (1982), who also found that ruminal butyrate does not affect the efficiency of N utilization in sheep. When compared to Na-Ac infusion, the proportion of urea-N partitioned to the GIT that was captured for microbial protein synthesis (i.e., GER to UUA) was increased by butyrate infusion. Thus, butyrate may be acting via other mechanisms (e.g., microbial dynamics, rumen NH_3 fluxes) to enhance capture of recycled urea-N in the rumen. Obviously, the mechanisms regulating movements of N into and out of the rumen are complex, and that multiple factors determine the net capture of recycled urea-N. It is also probable that acetate leads to inefficient use of N. More AA would need to be catabolized to feed into the Krebs's cycle (anaplerotic reactions) to metabolize acetate. An interesting approach to further investigate these N dynamics would be to combine butyrate infusion with addition of more fermentable carbohydrates to both enhance microbial protein synthesis and improve overall capture of recycled urea-N. Furthermore, butyrate was infused constantly in the current studies, which deviates from the more natural patterns of eating and fermentation. Intermittent feeding may result in a larger response in protein accretion (El-Kadi et al., 2012), and incorporation of butyrate in the feed might provide a more synchronous delivery with the needs of energy for digestion.

CHAPTER 4: ROLE OF RUMEN PROPIONATE IN REGULATION OF UREA NITROGEN KINETICS AND GLUCONEOGENESIS IN GROWING SHEEP

Abstract

Feeding and post-ruminal infusion of propionate is known to increase N retention in growing ruminants. The aim of this study was to determine the role of rumen propionate in regulation of urea-N recycling and gluconeogenesis in growing sheep. In Exp 1, wether sheep (n=6, 32.5 ± 3.57 kg BW), fitted with a rumen cannula, were fed to 1.8 × maintenance energy intake a pelleted concentrate-type ration (170 g CP/kg, 9.3 MJ ME/kg) and continuously infused into the rumen with iso-energetic (1 MJ/d) solutions of either Na-Acetate (control) or Na-Propionate for 9-d periods in a balanced crossover design. In Exp 2, a different group of wether sheep (n=5, 33.6 ± 3.70 kg BW), also fitted with a rumen cannula, were fed to >1.4 × maintenance energy intake and on an equivalent protein intake basis either a control (104 g CP/kg, 7.34 MJ ME/kg) or sodium propionate supplemented (100 g CP/kg, 7.75 MJ ME/kg) ration at 2-h intervals via an automatic feeder. [$^{15}N_2$]Urea was continuously infused i.v. for the last 5 d of each period, and total urine collected by vacuum and feces by a harness bag. Over the last 12 h, [$^{13}C_6$]glucose was continuously infused i.v. and hourly blood samples collected during the last 5 h. Propionate treatments increased ($P < 0.001$) the proportion of rumen propionate in both experiments. All animals were in positive N balance (4.34 g N/d in Exp 1; 2.0 g N/d in Exp 2). In Exp 1, N retention was not affected by propionate infusion as compared to iso-energetic acetate. There was no effect on urea entry (synthesis) rate

(UER) or urea-N recycling in Exp 1, however, Na-Propionate infusion tended ($P < 0.1$) to increase urinary urea-N excretion (UUE). In Exp 2, feeding propionate increased ($P < 0.01$) N retention by ~50%. In addition, UER was reduced by ~2 g urea-N/d, leading to a reduction ($P < 0.05$) in UUE (7.0 vs 6.2 g urea-N/d). Between the two experiments, the proportion of urea-N recycled to the gut was higher with the forage-type diet in Exp 2 (~60%) compared to the concentrate-type diet in Exp 1 (~40%), although urea-N fluxes across the rumen remained unchanged. Glucose entry and gluconeogenesis were higher ($P < 0.05$) and plasma glucose tended ($P < 0.1$) to be higher in Exp 1 with Na-Propionate infusion, but there was no change in Cori cycling. In Exp 2, glucose entry, gluconeogenesis, Cori cycling and plasma glucose increased ($P < 0.05$) with dietary propionate. Under the feeding and propionate supplementation conditions of these studies, higher ruminal propionate does not affect urea-N fluxes across the rumen.

Introduction

Feeding highly rumen fermentable diets increases urea-N recycled to the rumen (Kennedy and Milligan, 1980; Huntington, 1989) and increases the capture of rumen NH_3 by microbes (Stern and Hoover, 1979) leading to lower urinary N losses and hence, greater N retention (Fluharty et al., 1999). However, the mechanisms which dictate urea-N kinetics are not well elucidated. Rumen volatile fatty acids (VFA) are a primary product of rumen fermentation, and are responsible for meeting ~70-80% of the animal's energy needs (Bergman and Wolff, 1971). Among the VFA, propionate, which makes up about 15-40% of the total rumen VFA (Bergman, 1990), may play a key role in regulation of urea-N kinetics and utilization, as suggested by several studies. Higher rumen propionate resulting from feeding highly fermentable diets is associated with

greater N retention (Abdul–Razzaq and Bickerstaffe, 1989; Obara and Dellow, 1994). Feeding and post-ruminal infusion of propionate are also known to increase N retention in growing ruminants, possibly through increasing urea-N recycling and/or gluconeogenesis (Kim et al., 1999; Baldwin et al., 2012). However, there appears to be an interaction between feed composition (starch vs fiber) and propionate supplementation on N retention (Moloney, 1998).

Addition of propionate to the ruminant ration may improve protein efficiency by several plausible mechanisms. Ruminal propionate enhances epithelial proliferation (Sakata and Tamate, 1979), and is the main substrate for gluconeogenesis (Bergman, 1990). Thus, propionate may spare amino acids (AA) for energy and gluconeogenesis, hence reducing AA catabolism, and urea synthesis in the liver. Propionate has also been shown to inhibit synthesis of N-acetyl glutamate (Stewart and Walser, 1980), the allosteric regulator of hepatic carbamoyl-phosphate synthetase-I, resulting in a decrease in urea cycle activity and AA catabolism in the liver. Abdul-Razzak (1989) showed that diets resulting in a higher propionate-type fermentation stimulates release of insulin and increased muscle protein synthesis. However, when propionate was delivered intra-ruminally, no effect was found on hind limb AA balance (Ortigues-Marty, 2003). In this respect, despite indirect and direct evidence of the role of propionate on N utilization in ruminants, the underlying mechanisms remain unresolved.

To answer these questions., we conducted two experiments in growing sheep. In Exp 1, iso-energetic infusions of acetate and propionate were used to simulate comparisons of a high forage and a high concentrate diet in terms of rumen fermentation profiles. In Exp 2, forage-type diets with or without added propionate were compared to

directly determine the role of propionate in regulation of urea-N kinetics under typical feeding conditions where rumen VFA concentrations fluctuate.

Materials and methods

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland (protocol numbers R-10-45 and R-11-33).

Animals, diets and treatment periods

Experiment 1

Upon arrival in the ANSC animal facilities, Polypay × Dorsett wether lambs (32.5 ± 3.57 kg BW) were transitioned to a standard pelleted diet (**Table 4.1**) and fed to 1.8 × maintenance energy (ME) requirements. After acclimation and a 30-d quarantine period, sheep were surgically fitted with a rumen cannula (Appendix 1). Following recovery from surgery, sheep were placed into individual metabolic crates and fed an equal amount of the basal diet every 2-h via an automatic feeder. Six cannulated sheep were assigned to receive iso-energetic (~1 MJ/d based on gross energy of acetate and propionate) rumen infusions of either sodium acetate (Na-Acetate) or sodium propionate (Na-Propionate) in a balanced cross-over design with each infusion period lasting 9 d. The propionate infusion rate was calculated to supply 10% additional ME (~1 MJ/d) and to increase rumen propionate concentration by ~20% as was observed by Moloney (1998). The infusates were adjusted to pH 6.8. Each infusion period was separated by 5 d during which sheep were placed into individual floor pens for exercise and to allow a period of treatment washout.

Table 4.1: Composition of diet for Exp 1.

Item	Amount, g/kg (as fed)
Ingredient	
Ground Corn	385
Alfalfa	540
Soybean Meal	50
Vitamin-mineral premix[1]	25
Chemical composition (g/kg DM)	
DM	886
CP	172
Soluble Protein	49
ADF	192
NDF	298
Starch	294
Crude fat	33
TDN	689
N_{em}, MJ/kg	6.64
N_{eg}, MJ/kg	4.15

[1]Bel Air sheep mineral vitamin mixture (per kg of premix): Ca, 210 g; P, 30 g; NaCl, 160 g; S, 30 g; Mg, 25 g; K, 24 g; Fe, 2.4 g; Mn, 2.4 g; Zn, 2.7 g; Se, 24 mg; Co, 30 mg; I, 40 mg; Choline-Cl, 4.41 g; vitamin A, 661,500 IU; vitamin D_3 132,300 IU; vitamin E, 1,764 IU.

DM: dry matter; CP: crude protein; ADF: acid detergent fiber; NDF: neutral detergent fiber; TDN: total digestible nutrients

Experiment 2

Five Polypay × Dorsett wether lambs (33.6 ± 3.70 kg BW) were transitioned to a forage-type diet (**Table 4.2**) and fed to 1.4 × ME requirements. The sheep were similarly fed and prepared with rumen cannulas as in Exp 1. Following recovery from surgery, sheep were assigned to receive either the control diet or the control diet supplemented with sodium propionate (**Table 4.2**) in a balanced cross-over design with each period lasting 9 d. During the last 5 d, sheep were placed into individual metabolic crates for N balances, urea-N kinetics and gluconeogenic measurements (see below). Each period was separated by 5 d for washout.

Tracer infusion

Temporary jugular vein catheters were inserted at least 2 d prior to initiating isotope infusions. Over the last 5 (Exp 1) or 4 (Exp 2) days of each treatment period a sterile solution containing [$^{15}N_2$]urea (99 atom percent ^{15}N, Cambridge Isotope Laboratories Inc., Andover, MA) was continuously infused (0.6 g/d) through a jugular vein catheter. It has been shown that this length of [$^{15}N_2$]urea infusion results in the attainment of isotopic plateau in urinary [$^{15}N^{14}N$] and [$^{15}N^{15}N$]urea enrichment and that this infusion rate increases the urinary [$^{15}N^{15}N$]urea enrichment to 1.5-2.5 atoms percent excess (APE) (Sunny et al., 2007). Glucose kinetics were measured by infusion of [$^{13}C_6$]glucose. On the last day of each experimental period, an *i.v.* bolus (priming) dose (0.45 g) of [$^{13}C_6$]glucose was administered followed by continuous infusion (0.15 g/h) for 9 h.

Table 4.2: Composition of diets for Exp 2.

	Control	Dietary Propionate
Ingredient (g/kg), as fed		
Ground Corn	53	50
Timothy hay-sun cured	890	846
Soypass	50	50
Sodium Propionate		47
Vitamin-mineral premix[1]	7.5	7.5
Chemical composition (g/kg DM)		
DM	874	864
CP	151	139
Soluble Protein	36	24
ADF	323	299
NDF	516	510
Starch	89	85
Crude fat	23	24
TDN	565	557
N_{em}, MJ/kg	4.98	4.89
N_{eg}, MJ/kg	2.58	2.49

[1]Bel Air sheep mineral vitamin mixture (per kg of premix): Ca, 210 g; P, 30 g; NaCl, 160 g; S, 30 g; Mg, 25 g; K, 24 g; Fe, 2.4 g; Mn, 2.4 g; Zn, 2.7 g; Se, 24 mg; Co, 30 mg; I, 40 mg; Choline-Cl, 4.41 g; vitamin A, 661,500 IU; vitamin D_3 132,300 IU; vitamin E, 1,764 IU.

DM: dry matter; CP: crude protein; ADF: acid detergent fiber; NDF: neutral detergent fiber; TDN: total digestible nutrients

Sampling

During the last 5 d of each treatment period, sheep were fitted with a harness for total collection of feces (by bag) and urine (by suction), which were weighed and recorded. A slight vacuum was used to collect urine directly into a sealed container placed on a stir plate and containing sufficient HCl to reduce urine pH to < 3 to prevent bacterial hydrolysis of urea and ammonia volatilization. Well-mixed sub-samples from each collection (100 g feces and 40 g urine) were stored at -20^{o}C for later analysis. Over the last 6 h of tracer infusion, urine samples were also collected at 2 h intervals and later analyzed to verify that plateau of [$^{15}N_2$]urea enrichment in urine had been attained. Hourly blood samples were collected during the last 5 h. Plasma was separated by centrifugation (1,000 × *g* for 15 min at 4^{o}C) and stored at -20^{o}C for later analysis. At the end of each experimental period, following collection of the last sample, sheep were removed from crates and within 10 min the rumen was completely evacuated by vacuum. The rumen fluid was mixed well and a representative sample (50 mL) strained through two layers of cheese cloth. The strained rumen fluid was immediately centrifuged (1,000 × *g* for 10 min at 4^{o}C) and the pH of the supernatant recorded using a pH indicator strip (VWR International, West Chester, PA). For determination of VFA concentration, to a known amount (1 g) of rumen fluid was added a known weight (0.25 g) of an internal standard mixture (260 mM [1-^{13}C]acetate, 80 mM [methyl-D_3]propionate and 40 mM [1-^{13}C]butyrate), and the samples stored at -20^{o} C for later analysis.

Urea concentration and enrichment

The concentration and enrichment of plasma and urinary urea were determined by gas chromatography-mass spectrometry (GC-MS, Agilent 6890 series gas chromatography system coupled to an Agilent 5973N mass selective detector; Agilent; Palo Alto, CA) under electron ionization (EI) mode. For determination of urinary urea concentration, to a known weight (0.25 g) of an internal standard solution containing [^{13}C, $^{15}N_2$]urea (5 mg/g) was added an equal known weight of urine. For plasma urea concentration, a known amount (0.5 g) of internal standard containing [^{13}C, $^{15}N_2$]urea (0.26 mg/g) was added to an equal known weight of plasma. Plasma and urine samples were acidified by adding an equal volume of 15% sulfosalicylic acid or 10% trichloroacetic acid (w/v), respectively, and centrifuged for 10 min at 10,000 × *g* at room temperature to precipitate proteins and other debris. Urea was isolated from acidified samples by application to 0.5 g of cation exchange resin (AG 50W-X8 resin, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA). The resin was washed with 2 × 2 mL of water and urea eluted with 2 mL of ammonium hydroxide plus 1 mL of double distilled water. An aliquot (100 to 200 µl) of the elute was dried under N_2 gas and the tertiary-butyldimethylsilyl derivative of urea was prepared by adding 50 µl each of acetonitrile (Pierce chemicals , Rockford, IL) and N-methyl-N-t-butyl-dimethylsilyl-trifluroacetamide (MTBSTFA, Pierce chemicals , Rockford, IL) followed by heating at 90^{o}C for 20 min (El-Kadi et al., 2006). Derivatized urea samples were separated on a fused silica capillary column (HP-50; 30 m × 0.25 mm × 1 µm Hewlett-Packard, Palo Alto, CA) prior to MS under EI conditions. Ions of mass-to-charge (m/z) 231.2 (unlabeled; M0), 232.2 (singly labeled; [M+1]), 233.2 (doubly labeled; [M+2]), and

234.2 (internal standard; [M+3]) were monitored. The GC inlet was set at 250 ^{o}C and the GC conditions were: initial temperature of 150^{o}C followed by 15^{o}C/min to 250^{o}C. Urea concentration in the samples was calculated by isotope dilution (Calder et al., 1999) based on the ratios of [M+3]:[M0]urea after correction for background (natural abundance) and spillover effects of the enriched [M+2]urea.

Total urinary and fecal N, and fecal ^{15}N

Total urinary and fecal N were measured using an automated N analyzer (CN-2000, Leco, St. Joseph, MI). Fecal samples were dried in a forced-air drying oven at 60^{o}C for 5 d. Dried samples were pulverized in a liquid nitrogen freezer mill (Freezer-Mill 6850, Spex CertiPrep Inc., Metuchen, NJ) and samples submitted to the stable isotope facility at the University of California-Davis (CA) for ^{15}N analysis by isotope-ratio mass spectrometry.

Rumen fluid VFA concentration

Samples were thawed and acidified by adding 250 µL of 2 M HCl, and centrifuged for 15 min at 13,000 × *g* at room temperature to precipitate solids. The supernatant was filtered through a 0.2 µm filter (Nalgene, Rochester, NY), and anhydrous ether (0.5 mL) added to extract VFA. An aliquot (200 µl) of extracted VFA was converted to the tert-butyldimethylsilyl derivative by adding 50 µL MTBSTFA followed by heating at 80^{o}C for 20 min (Duncan et al., 2004). After samples had cooled, 50 µL of methanol was added to react with the surplus MTBSTFA. Derivatized samples were injected onto the GC column (fused silica capillary column, HP-5; 30 m × 0.25 mm × 1 µm Hewlett-Packard) with the GC inlet set at 250°C and the following columns

conditions: initial temperature of 60°C held for 3 min followed by 10°C/min to 210°C for 4 min. Ions of m/z 117 and 118 for acetate, 131 and 134 for propionate and 145 and 146 for butyrate were monitored. VFA concentrations were calculated by isotope dilution (Calder et al., 1999).

Glucose concentration and enrichment

For determination of plasma glucose concentration, a known amount (0.5 g) of an internal standard containing [$^{13}C_6$; 1, 2, 3, 4, 5, 6, 6-$^{2}H_7$]glucose (4 mM in 0.1 M HCl) was added to an equal known weight of plasma. The samples were acidified with an equal volume of 15% sulfosalicylic acid (w/v) and centrifuged for 10 min at 10,000 × *g* at room temperature to precipitate proteins. The supernatant was applied to 0.5 g cation-exchange resin (AG 50W-X8 resin, 100-200 mesh) and the glucose containing fraction eluted with 2 mL distilled water, frozen and lyophilized to dryness. Glucose was converted to the di-O-isopropylidene derivative for GC-MS analysis (Hachey et al., 1999). Briefly, to the freeze dried sample was added 1 mL of freshly prepared 0.38 M sulfuric acid in acetone. The solution was incubated for 1 h at room temperature after which it was neutralized by the addition of 3 mL of 0.44 M sodium carbonate. After the addition of 3 mL of saturated NaCl, the glucose derivative was back extracted with 3 mL of ethyl acetate. The upper ethyl acetate phase was dried under N_2 gas. Next, the di-O-isopropylidene derivative was acetylated by the addition of 50 µL each of ethyl acetate and acetic anhydride followed by heating for 30 min at 60°C. The glucose derivative was separated on a fused silica capillary column (HP-5; 30 m × 0.25 mm × 1 µm, Hewlett-Packard) prior to MS under EI conditions. The GC inlet was set at 250°C and the following columns conditions: initial temperature of 80°C followed by 10°C/min to

260^{o}C. Ions of m/z 287 to 293 (glucose isotopomers [M0] to [M+6]) and 300 (internal standard; [M+13]) were monitored. Glucose concentration in samples was calculated by isotope dilution (Calder et al., 1999) based on the ratio of [M+13]:[M0]glucose after correction for background (natural abundance), and corrected for the concentration of [M+1]-[M+6]glucose isotopomers. The enrichments of plasma glucose were corrected for natural abundance using a matrix approach (Fernandez et al., 1996).

Calculations

Urea N kinetics: Urea-N kinetic calculations were based on Lobley et al. (2000). Whole body urea synthesis (urea entry rate; UER) was estimated from the dilution of the infused [$^{15}N_2$]urea tracer. A portion of UER is excreted into urine (UUE), whereas the remainder enters the gut tissues (GER). Urea entering the gut has three different fates: 1) excretion in feces (UFE), 2) hydrolysis by rumen microbes with absorption of NH_3 and return to the ornithine-urea cycle (ROC), and 3) utilization by gut microbes for protein synthesis (anabolic use, UUA). UFE and ROC are estimated directly from excretion of fecal ^{15}N and based on the appearance of urinary [$^{15}N^{14}N$]urea, respectively. UUA is estimated by the difference between GER and UFE+ROC. An important assumption in this model is that all the doubly labeled urea in urine is derived from the infused tracer. Calculations for urea-N kinetics are shown in Appendix 3.

Gluconeogenesis: Gluconeogenesis and glucose recycling (Cori cycling) were estimated using corrected mass isotopomer distribution (MID) enrichments according to a method described by Tayek and Katz (1997), where:

Glucose entry (g/d) = (([$^{13}C_6$]glucose tracer purity (%) / [M+6]glucose) – 1) × [$^{13}C_6$]glucose infusion rate (g/d)

Cori cycling (g/d) = ([M+1] + [M+2] + [M+3]) / ([M+1] + [M+2] + [M+3] + [M+6]) × glucose entry (g/d)

Gluconeogenesis, i.e. gluconeogenesis from non-glucose sources, was calculated by difference as,

Gluconeogenesis (g/d) = Glucose entry - Cori cycling

Glucose entry represents the appearance in blood of glucose derived from absorption and from synthesis in the body. However, because dietary starch is extensively fermented by rumen microbes and the fact that nearly all glucose absorbed across the intestines is metabolized, it is safe to assume that net glucose absorption across the gut is negligible (El-Kadi et al., 2006). Thus, glucose entry largely reflects Cori cycling and gluconeogenesis.

Statistical Analysis

Animals were nested within groups. Treatment and period were considered to be fixed effects and animals within group were considered to be random effects. Data were analyzed using the PROC MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC).

The following linear model was used to analyze the data:

$$Y_{ijk} = \mu + group_i + animal_{ij} + period_k + treatment_h + error_{ijk}$$

where,

Y_{ijk} = response variable (UER, UUE etc.) in the k^{th} period of the j^{th} animal in the i^{th} group (i = 1, 2; j = 1 to n; k = 1, 2)

μ = the overall mean effect

$group_i$ = the effect of the i^{th} group (i = 1,2)

$animal_{ij}$ = the effect of the j^{th} animal on the i^{th} group (j = 1, 2, .. , n_i)

$period_k$ = the effect of the k^{th} period (k = 1,2)

$treatment_h$ = the effect of the h^{th} treatment (h = 1,2; being a function of i & k)

$error_{ijk}$ = the residual error

Statistical significance was set at $P \leq 0.05$ and a trend at $P \leq 0.10$.

Results

Experiment 1: Na-Propionate vs Na-Acetate (control)

Feed intake, digestibility and N-retention: There were no feed refusals and all animals gained weight during the experiment. Dietary DM (66 to 72%) and N (62 to 72%) digestibility did not differ between treatments (**Table 4.3**). There was no effect of Na-Propionate infusion on N retention, and urinary and fecal N outputs compared to Na-Acetate.

Table 4.3: Nitrogen balance in growing sheep in Exp 1 (n=6) and Exp 2 (n=5).

	Exp 1				Exp 2			
	Na-Acetate (control)	Na-Propionate	SE	*P*	Control	Dietary Propionate	SE	*P*
N balances[1] (g N/d)								
N intake	23.62	22.99	0.91	NS	23.55	23.15	0.84	0.051
N in urine	11.43	11.95	0.38	NS	14.38	13.02	0.59	0.048
N in feces	7.39	7.16	0.17	NS	7.54	7.74	0.29	NS
N retained	4.81	3.88	0.80	NS	1.63	2.39	0.10	0.006
DM digestibility (%)	69.47	69.04	0.57	NS	55.78	54.74	0.99	NS
N digestibility (%)	68.69	68.71	1.06	NS	68.15	66.48	0.55	0.078
Urine urea-N:total urine N	0.76	0.81	0.02	NS	0.49	0.48	0.01	NS
N retained:N digested	0.29	0.24	0.04	NS	0.10	0.16	0.01	0.009

[1]Nitrogen balance was measured during the last 4 d of each 9 d treatment period.

Rumen pH and VFA profile: Compared to Na-Acetate (control), infusion of Na-Propionate reduced ($P < 0.05$) rumen pH (6.5 vs 6.2). Both the concentration and molar proportion (mol/100 mol VFA) of rumen propionate and butyrate were higher ($P < 0.001$) and that of acetate lower ($P < 0.001$) when Na-Propionate was infused (**Table 4.4**).

Urea-N kinetics: For all treatments, urinary [M+2]urea enrichment reached an isotopic plateau (**Figure. 4.1**) within 2 d of [$^{15}N_2$]urea infusion. Urinary [M+1]urea enrichment increased until attaining a plateau between 2 and 3 d of infusion (**Figure. 4.2**). Total fecal ^{15}N enrichment continued to increase throughout the infusion period (**Figure. 4.3**), attaining a final value approximately 80% of predicted plateau. Hence, the fecal ^{15}N enrichment on the last day was used in calculations. Urea-N kinetics are shown in **Table 4.5**. There was no change in UER, however, UUE tended ($P < 0.1$) to increase and UFE decrease ($P < 0.05$) with Na-Propionate infusion. Plasma urea concentration was higher ($P < 0.05$) with Na-Propionate infusion.

Glucose entry and gluconeogenesis: Glucose entry and gluconeogenesis increased ($P < 0.05$) with infusion of Na-Propionate (**Table 4.6**), but there was no difference in Cori cycling. Plasma glucose concentration tended ($P < 0.1$) to be higher with Na-Propionate infusion. The molar conversion efficiency of Na-propionate to gluconeogenesis was ~30%.

Table 4.4: Rumen volatile fatty acid profiles in growing sheep in Exp 1 (n=6) and Exp 2 (n=5).

	Exp 1				Exp 2			
	Na-Acetate (control)	**Na-Propionate**	**SE**	***P***	**Control**	**Dietary Propionate**	**SE**	***P***
Concentration (mM)								
Acetate	132.3	108.9	9.57	0.017	68.8	74.7	4.16	NS
Propionate	18.2	41.3	2.16	0.001	17.3	30.6	2.02	0.004
Butyrate	10.7	19.2	1.57	0.007	13.2	12.4	1.60	NS
Molar proportion (mol/100 mol)[1]								
Acetate	81.8	64.4	1.31	<.0001	69.5	63.6	1.06	0.016
Propionate	11.6	24.7	0.98	<.0001	17.3	25.9	0.73	<0.001
Butyrate	6.6	11.0	0.64	0.001	13.1	10.6	0.81	0.013
pH	6.5	6.2	0.08	0.014	6.6	6.9	0.10	NS

[1] Moles of each VFA relative to the sum of acetate, propionate and butyrate.

Table 4.5: Urea-N fluxes in growing sheep in Exp 1 (n=6) and Exp 2 (n=5).

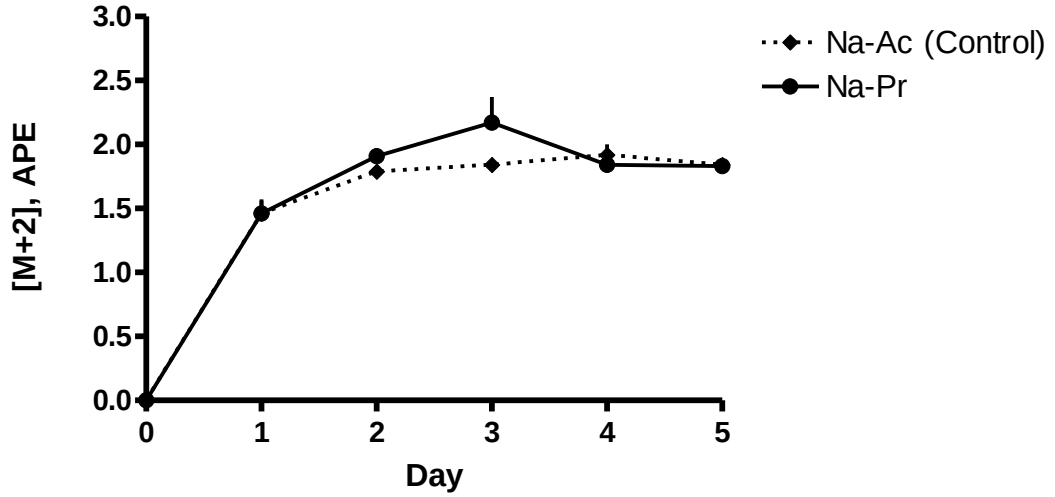
	Exp 1				Exp 2			
	Na-Acetate (control)	Na-Propionate	SE	*P*	Control	Dietary Propionate	SE	*P*
Urea flux (g N/d)								
UER	16.7	17.3	0.45	NS	17.2	15.1	0.80	0.054
UUE	8.7	9.7	0.29	0.066	7.0	6.2	0.30	0.042
GER	8.0	7.6	0.68	NS	10.2	8.9	0.59	NS
ROC	5.0	4.5	0.79	NS	7.3	6.3	0.38	NS
UFE	1.4	1.0	0.09	0.029	0.9	1.1	0.06	0.076
UUA	1.5	2.1	0.58	NS	2.0	1.5	0.35	NS
Fractional transfers								
UER to urine (u)	0.53	0.56	0.03	NS	0.41	0.41	0.01	NS
GER to ROC (r)	0.63	0.56	0.09	NS	0.72	0.71	0.02	NS
GER to feces (f)	0.19	0.14	0.01	0.038	0.09	0.12	0.01	0.035
GER to UUA (a)	0.19	0.31	0.10	NS	0.18	0.17	0.03	NS
Plasma urea (mM)	3.40	4.08	0.18	0.026	4.68	3.54	0.19	0.010

UER = urea entry rate; UUE = urinary urea elimination; GER = gut entry rate; ROC = return to ornithine cycle; UFE = urea-N fecal elimination ; UUA = urea-N used for anabolism.

Table 4.6: Glucose kinetics in growing sheep in Exp 1 (n=6) and Exp 2 (n=5).

	Exp 1				Exp 2			
	Na-Acetate (control)	**Na-Propionate**	**SE**	***P***	**Control**	**Dietary Propionate**	**SE**	***P***
Glucose kinetics (g/d)								
Glucose entry	121.6	140.6	4.79	0.026	89.9	114.5	2.37	0.003
Cori recycling	41.3	41.9	3.86	NS	26.9	33.2	0.83	0.002
Gluconeogenesis	80.2	98.6	2.86	0.010	63.0	81.3	2.00	0.002
Plasma glucose (mM)	3.8	4.1	0.11	0.080	3.9	4.3	0.13	0.029

A)



B)

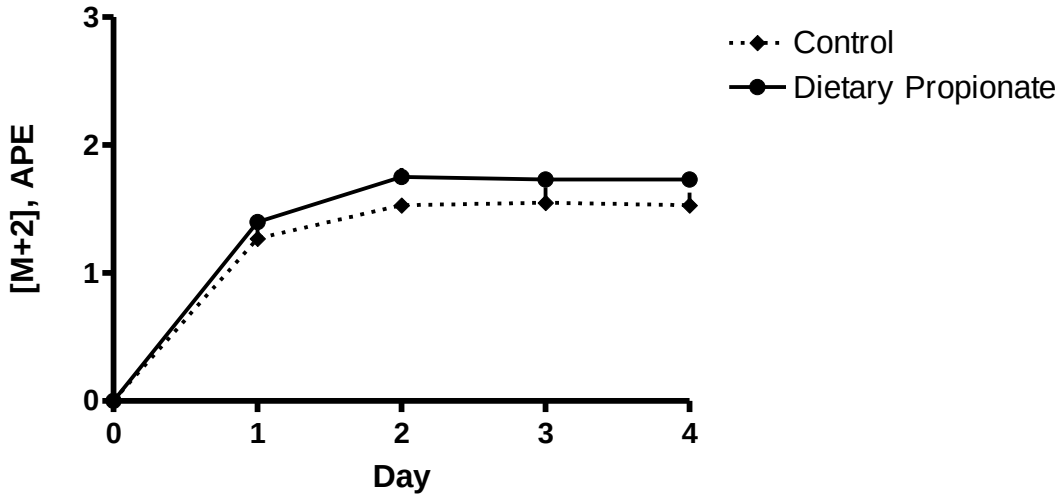
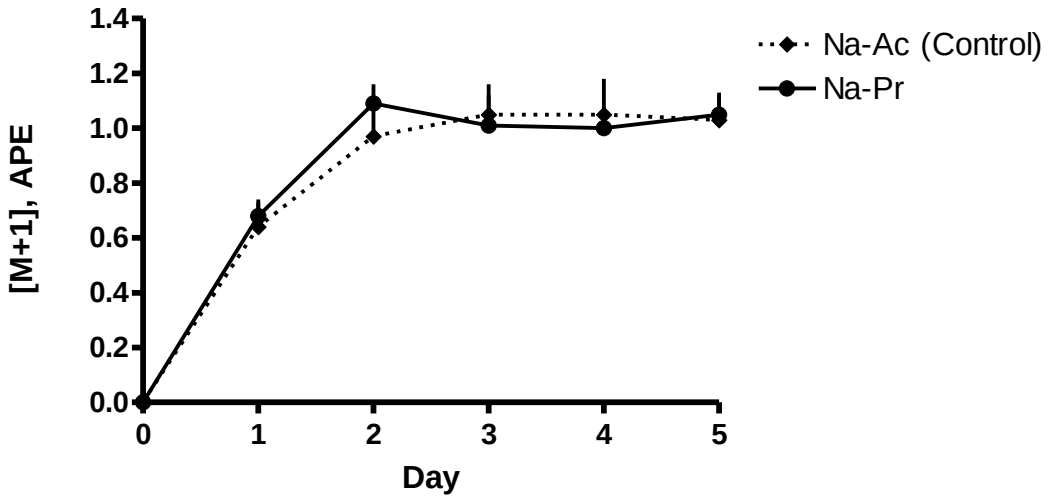


Figure 4.1: The time-course of urinary [$^{15}N^{15}N$]urea enrichments in Exp 1 (A, 5 day [$^{15}N_2$]urea infusion) and Exp 2 (B, 4 day [$^{15}N_2$]urea infusion). Values are the mean (± standard error).

A)



B)

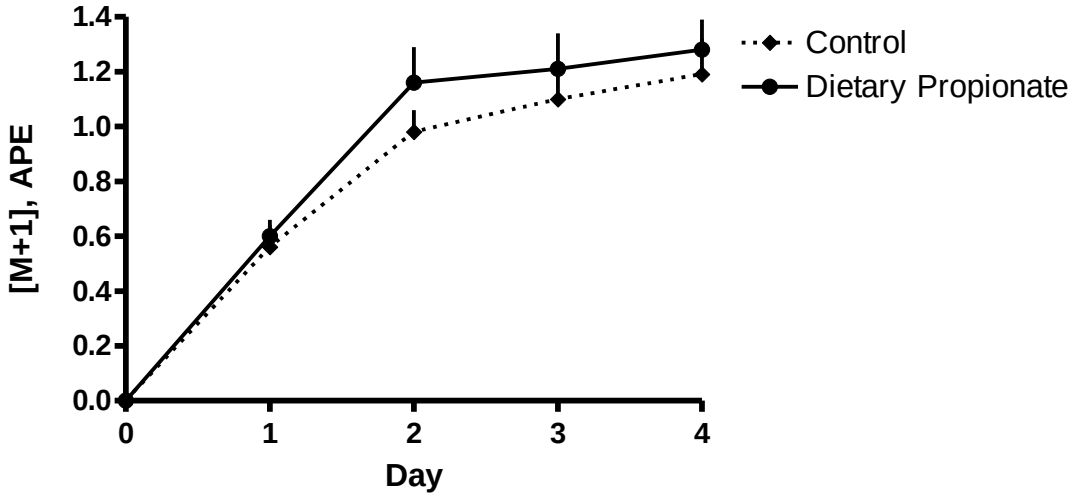


Figure 4.2: The time-course of urinary [$^{15}N^{14}N$]urea enrichments in Exp 1 (A, 5 day [$^{15}N_2$]urea infusion) and Exp 2 (B, 4 day [$^{15}N_2$]urea infusion). Values are the mean (± standard error).

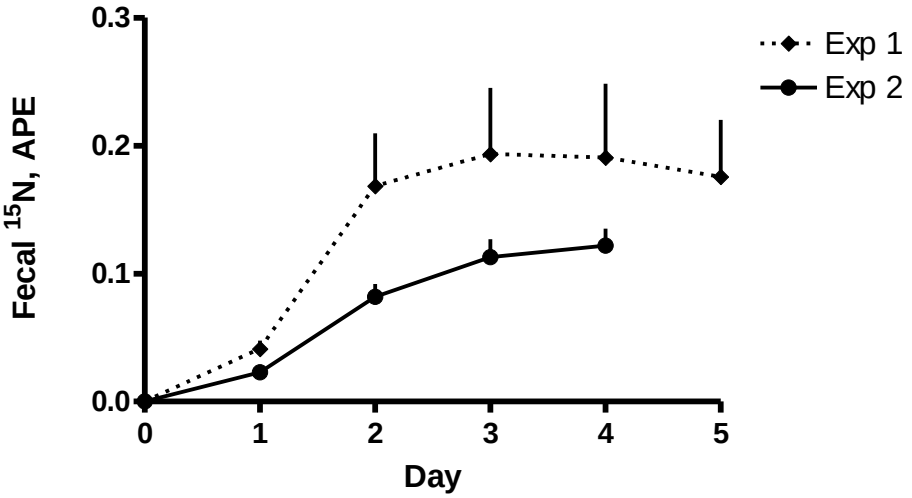


Figure 4.3: Time-course of total ^{15}N enrichment in fecal samples in Exp 1 (5 day [$^{15}N_2$]urea infusion) and Exp 2 (4 day [$^{15}N_2$]urea infusion). Values are the mean (± standard error)

Experiment 2: Control diet vs Dietary Propionate

Feed intake, digestibility and N balances. All animals gained weight during the experiment. Dietary DM digestibility (52 to 58%) was not affected between treatments, however, N digestibility (65 to 69%) tended ($P < 0.1$) to be reduced when the propionate diet was fed (**Table 4.3**). Total N intake was only marginally different, owing to variation in diet composition. The propionate diet reduced ($P < 0.05$) urinary N excretion and thus increased ($P < 0.01$) N retention. No difference was observed in fecal N output.

Rumen pH and VFA profiles. Rumen pH was not altered by treatments (**Table 4.4**). The molar proportion of rumen propionate was higher ($P <0.001$) while that of butyrate and acetate were lower ($P < 0.05$) with the propionate diet. However, the rumen

concentrations of propionate were increased ($P < 0.01$) with the propionate diet with no changes in acetate or butyrate (**Table 4.4**).

Urea-N kinetics. For all treatments, urinary [M+2]urea enrichment reached an isotopic plateau (**Figure 4.1**) within 2 d of [$^{15}N_2$]urea infusion. Urinary [M+1]urea enrichment increased until attaining a plateau between 2 and 3 d of infusion (**Figure 4.2**). Total fecal ^{15}N enrichment continued to increase throughout the infusion period (**Figure. 4.3**), attaining a final value approximately 80% of predicted plateau. Hence, the fecal ^{15}N enrichment on the last day was used in calculations. Urea-N kinetics are shown in **Table 4.5**. The propionate diet reduced ($P \leq 0.05$) UER and UUE. There was no difference in GER, ROC, and UUA; however, UFE tended ($P < 0.1$) to be increased with propionate diet. Plasma urea concentration was lower ($P < 0.05$) with propionate diet.

Glucose entry and gluconeogenesis. Glucose entry, Cori cycling and gluconeogenesis were greater ($P < 0.01$) with the propionate diet (**Table 4.6**). Plasma glucose also increased ($P < 0.05$) with propionate diet. The molar conversion efficiency of Na-Propionate in the diet to gluconeogenesis ~30%.

Using combined data from both experiments, there was a significant correlation observed between rumen propionate concentration (x, mM) and gluconeogenesis (y, g/d): $y = 0.9322x + 55.413$, $R^2 = 0.42$, $P < 0.001$ (**Figure 4.4**).

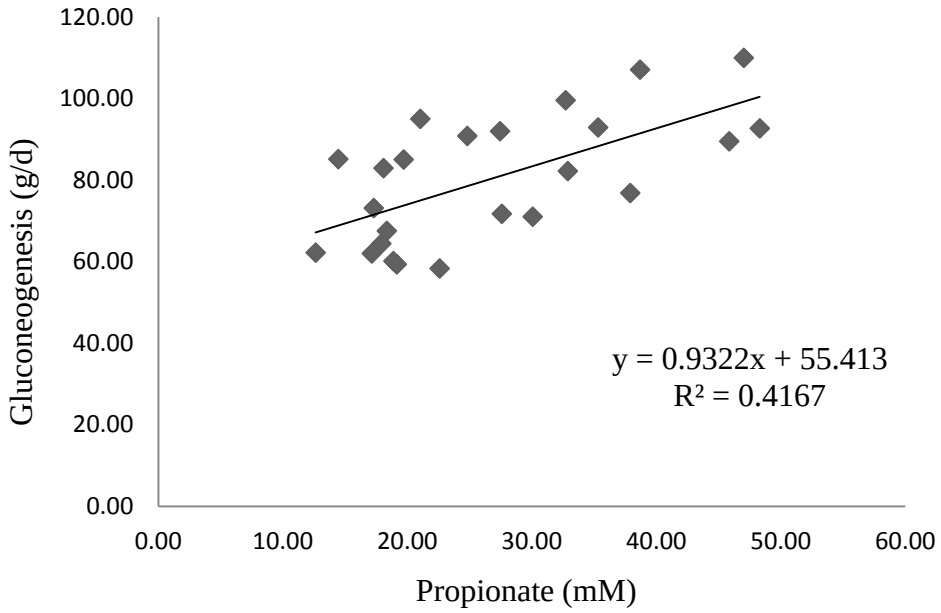


Figure 4.4: Relationship between rumen propionate (mM) and gluconeogenesis (g/d).

Discussion

Higher concentrate diets lead to increased rumen propionate concentration, and often greater N retention in ruminants (Abdul–Razzaq and Bickerstaffe, 1989; Obara and Dellow, 1994). More direct evidence of the influence of propionate on growth and N economy of ruminants comes from studies where propionate was either fed or infused into the GIT. For example, adding propionate to the diet of steers and sheep increased body weight gain and N retention (Moloney, 1998; Baldwin et al., 2012). And, post-ruminal infusion of propionate increased N retention and urea-N recycling to and capture in the GIT of growing sheep (Kim et al., 1999). What remains unresolved from those studies is whether the effect of propionate on N retention is simply a response to additional energy and(or) whether propionate spared AA from catabolism for

gluconeogenesis with greater partition of AA into protein. Thus, the aim of the current studies was to directly establish the role of rumen propionate on N utilization, urea-N kinetics, and gluconeogenesis in growing sheep.

In Exp 1, rumen propionate was elevated by infusion of Na-Propionate into the rumen of growing sheep fed a concentrate-type diet (294 g starch/kg DM feed), and using Na-Acetate as an iso-energetic control. Thus, under these dietary conditions (high starch), we aimed to simulate a forage-fed (Na-Acetate) compared to a typical concentrate-fed (Na-Propionate) rumen VFA profile. Infusion of Na-Propionate, compared to Na-Acetate, led to changes in the rumen VFA profile reflective of feeding a high concentrate diet wherein propionate was elevated and acetate was reduced. Yet, despite a tendency for propionate to shift urea-N output from feces to urine, there was no difference in total urinary or fecal-N output. Thus, under these dietary conditions and with continuous ruminal infusion of Na-Acetate and Na-Propionate, there were no differences in N retention. It is noteworthy that rumen butyrate concentration was increased with infusion of Na-Propionate, which reflects the complex nature of rumen fermentation and the likelihood that rumen microbial populations may have been altered. We observed a similar effect in the previous studies (Chapter 3) where butyrate infusion into the rumen also elevated propionate concentration.

After failing to observe a response in a concentrate-type diet and using an iso-energetic control, a second experiment was conducted with a forage-type diet (85 to 89 g starch/kg DM). Using a nearly identical diet as herein and feeding at ~1.6 × ME intake requirement, it was previously shown that addition of propionate to the diet increased N-retention by 38% in growing steers (Baldwin et al., 2012). In Exp 2, propionate was

included in the diet at the same level as infused in Exp 1, i.e. propionate supplied 10% additional energy compared to the control diet. Feeding the propionate diet at 2-h intervals increased N-retention (~0.8 g N/d), and this was mostly due to a reduction in urinary-N excretion (~1.4 g urea-N/d). Furthermore, the efficiency of N utilization (i.e., N-retained:digestible-N) was increased by ~60% with the propionate diet.

N retention and urea-N kinetics

Kim et al. (1999) infused propionate into the abomasum (post-rumen) of sheep fed a forage-type diet and observed higher N-retention as a result of greater urea-N recycling to and capture in the GIT. By contrast, Seal and Parker (1996) did not observe an increase in net portal-drained viscera removal of urea when propionate was infused into the rumen of growing steers that had been fed a forage diet. Moreover, there may be an interaction between diet type (starch or forage) and propionate supplementation (Moloney, 1998). In our studies, Exp 1 was on a concentrate-type diet with high starch content, and no effect of Na-Propionate infusion on N-retention was observed. By contrast, in Exp 2, propionate addition to a forage-type diet resulted in ~60% increase in N retention on an apparent digested-N basis. Although N retention increased when feeding propionate, the level of N retention achieved in Exp 2 (1.6 to 2.4 g N/d) was lower than in Exp 1 (4.3 to 4.8 g·N/d). Nonetheless, assuming protein comprises 66% of tissue dry matter, the 1 g N/d (6.25 g protein) increase in N retention translates into an additional 9.5 g/d tissue weight gain. Furthermore, assuming that carcass tissue contains 25% DM, the increase in body weight gain in response to feeding propionate equates to 38 g/d. Greater N-retention by the sheep in Exp 1 was expected given the higher starch content of the diet and the resulting higher rumen VFA concentrations (169 to 171 mM)

compared the diet fed in Exp 2 that resulted in lower VFA concentrations (99 to 118 mM). A positive correlation between VFA supply and N retention has been demonstrated in sheep (Sun and Zhao, 2009), and it is well established that larger amounts of fermentable carbohydrates lead to higher N-retention (Obara and Dellow, 1994; Fujita et al., 2006).

In vitro studies have produced confounding results on the role of propionate in regulation of hepatic urea synthesis. Using slices of sheep liver, propionate was found to inhibit urea synthesis (Rattenbury, 1983). In rats, propionate was shown to inhibit the synthesis of N-acetyl glutamate (Stewart and Walser, 1980), the essential allosteric activator of the urea cycle enzyme carbamoyl phosphate synthetase I. However, with isolated sheep hepatocytes, propionate was found to promote urea production (Demigné et al., 1991), whereas in isolated rumen epithelial and duodenal mucosal cells there was no effect of propionate on urea synthesis compared to acetate (Oba et al., 2004). In Exp 1, Na-Propionate infusion had no effect on UER nor on urea-N recycling kinetics compared to iso-energetic infusion of Na-Acetate as control. However, in Exp 2, feeding propionate reduced UER by ~2 g urea-N/d, leading to a reduction in urinary urea-N excretion. The proportion of urea-N recycled to the gut (GER:UER) was greater with the forage-type diet (~60%, Exp 2) compared to the concentrate-type diet (~40%, Exp 1), although urea-N fluxes across the rumen in these two experiments remained unaltered. These results suggest that the mechanism(s) that propionate influences N retention may be energetically driven, i.e. by sparing of AA. However, modulation of urea-N recycling by propionate cannot be ruled out given the changes we observed in UUE and UFE in Exp 1.

Gluconeogenesis

The role of propionate as a precursor for glucose synthesis in ruminants is well established (Bergman and Wolff, 1971; Danfær et al., 1995). In both of the present experiments, gluconeogenesis was increased by ~18 g/d with the propionate treatments. It is noteworthy that a similar response in gluconeogenesis to propionate supplementation was observed in both of the current experiments despite differences in the basal diets (forage-type vs concentrate-type) and the mode of propionate delivery (continuous infusion vs inclusion in diet). On a molar basis, the apparent conversion efficiency of supplementary propionate to glucose was ~30%. This is much lower than what would be expected (i.e. >90%), given that little propionate is metabolized by rumen tissues (Kristensen and Harmon, 2004) and the liver removes +80% of the portal vein supply of propionate (Berthelot et al., 2002) Thus, propionate most likely replaced AA catabolised for gluconeogenesis, resulting in the lower apparent efficiency of propionate conversion to glucose. The sparing of AA from gluconeogenesis likely accounted for the reduction in UER and thus the increase in N retention in Exp 2. By contrast, the lack of response in UER and N retention to infusion of Na-Propionate versus Na-Acetate in Exp 1 may be due to the constant infusion of treatments. Thus, unlike feeding propionate where the 2-h feeding interval is likely to initiate spikes in plasma insulin that promote anabolic responses (Sano et al., 1993), the constant infusion mode of treatments in Exp 1 would fail to elicit spikes in plasma insulin. In Exp 2, the higher plasma glucose concentration may also reflect the 2-h feeding mode, resulting in glucose stimulation of anabolism in peripheral tissues.

The role of higher ruminal propionate concentration, resulting from highly fermentable diets, on increased N retention is not clear. Our studies were designed to investigate the potential effects of ruminal propionate on urea-N kinetics and N utilization. Under the conditions of these studies, higher ruminal propionate did not affect urea-N fluxes across the rumen. However, it is difficult to attribute a particular mechanism of propionate action on N utilization in ruminants given the potential interactions that likely exist between type of feed (forage vs concentrate), mode (continuous infusion or incorporation into feed) and the site (ruminally or postruminally) of delivery of propionate. The pulsatile effect when feeding propionate may also have provided a synchronous delivery of propionate along with the needs of energy for digestion which likely stimulated higher spikes in plasma insulin to enhance protein accretion.

SUMMARY AND CONCLUSIONS

There have been few studies that have evaluated the role of various types of diets in promoting urea-N recycling and improving N utilization. However, when diets are fed that contain large amounts of fermentable material, there are dynamic changes in not only rumen VFA, but also rumen NH_3 concentration and the rumen microbiota. Thus, it becomes difficult to attribute the observed effects (i.e., N balance or urea-N kinetics) to particular VFA, metabolites, or other conditions in the rumen (e.g., microbial protein supply and pH). A more direct approach to determine the relationship between rumen VFA and the mechanisms affecting urea-N recycling and N utilization in the rumen is by artificially manipulating the rumen environment by infusion of individual or mixtures of VFA. In the design of experiments in this thesis, every effort was made to maintain balanced conditions (energetic, osmotic, natraemic and nitrogenous) between control and treatment groups.

The process of urea-N recycling in ruminants has been the subject of investigation for more than half a century. Although a wide range of factors are known to be associated with changes in urea-N recycling, the underlying mechanisms of action are still not well defined. In recent decades, use of stable isotope tracers has made it possible to measure and estimate urea-N kinetics. With the generation of experimental data with different feeding and ruminal conditions, it may be possible to accurately model and predict urea-N partitioning. Mechanistic knowledge of urea-N partitioning has the potential to reveal targets that can be manipulated to improve protein efficiency of ruminants, and hence, reduce N excretion to the environment.

The research in this thesis addressed two hypotheses with regard to the roles of rumen propionate and butyrate in improving N retention by enhancing urea-N recycled to the rumen for microbial protein synthesis. The results from these studies suggest that infusion of starch shifts urea-N excretion from urine to feces, with no effect on N retention. Continuous infusion of butyrate into the rumen redistributes urea-N fluxes, possibly by enhancing rumen epithelial growth, but N retention remained unchanged. And lastly, continuous infusion of propionate did not affect N retention nor urea-N kinetics; however, feeding propionate resulted in greater N retention as a result of reduced urea synthesis and excretion, possibly by propionate sparing AA from catabolism for gluconeogenesis.

APPENDICES

1. Surgery protocol for rumen cannulation

(from IACUC protocol # R-11-33)

Procedure: Surgical placement of a permanent barrel cannula into the rumen of sheep.

1. Sheep
    a. Wether sheep of uniform body weight (20 to 25 kg) will be selected from a certified local vendor with appropriate health and vaccination records. On arrival, sheep will be fed a standard pelleted diet in 2 equal portions per day to achieve normal rates of growth.
    b. Sheep will be brought into the facilities, placed in individual floor pens (1.5 m x 2.5 m) and acclimated to personnel and facilities prior to surgery. Previous experience indicates that handling (holding, stroking) of the animals prior to surgery makes them calmer and easier to handle for surgery and during experimentation.
    c. Sheep will be fitted with a permanent barrel cannula into the rumen
2. Preparation for surgery
    a. The barrel cannula and associated flanges are gas sterilized and surgical instruments and supplies (drapes, gowns, towels, gauze) are heat sterilized.
    b. Preparation of animal for surgery:
        i. Feed is withheld for three meals (i.e. morning of surgery, and the two meals the day before surgery) and water withheld from the

mid-afternoon the day prior to surgery. This will minimize bloating and regurgitation of digesta during surgery, and help reduce gut volume and space taken up in the abdominal cavity.

ii. Animal is transported from holding pen to the preparation room (0307).

iii. Wool is clipped (no 40 clipper blades) in the surgical field (most course wool will be clipped the day prior to surgery to minimize time spent in the surgery).

iv. The area over the jugular veins is clipped (no. 40 clipper blades), scrubbed with antiseptic soap and iodine, and the area flushed with 70% alcohol (ethanol).

v. A temporary catheter (Abbo-cath, 14 g, 5.5 in) is inserted into a jugular vein and secured with skin sutures.

vi. The pre-anaesthetics xylazine (0.2 mg/kg) and Butorphanol (Torbugesic, 0.1 mg/kg) are given i.v., and the catheter flushed with sterile saline.

vii. Immediately, the sheep is moved to the surgery suite and placed on the table in sternal recumbency.

viii. A gas mask is placed over the sheep's mouth, and isoflurane inhalation initiated (initially 4-5% isoflurane).

ix. The sheep is induced with Ketamine (5 mg/kg) and Diazepam (0.4 mg/kg).

x. Once adequately sedated, the head is extended forwards and gauze placed around the lower and upper jaws to hold mouth open for intubation.

xi. Lidocaine is lightly applied to the epiglottis to initiate opening of the airway. An endotracheal tube (7-9 mm i.d.) is then placed into the trachea, the cuff inflated, and the endotracheal tube secured in the mouth with gauze.

xii. The sheep is then maintained on isoflurane (1-2% isoflurane) and a fitted with a monitoring cuff around a front leg to allow for monitoring of heart rate and oxygen status, and breathing rate monitored. All parameters will be recorded at 5 min intervals throughout surgery.

xiii. The surgical field (left paracostal region) is scrubbed with antiseptic soap and iodine, and flushed with 70% alcohol (ethanol).

xiv. Peri-operatively, sheep are given the analgesic banamine (1.1-2.2 mg/kg, i.v.) and the antibiotic ceftiofur HCl (1-2 mg/kg, i.v.).

3. Surgery

i. The surgical field (approx. 2” posterior to the last rib and approx. 6” from the dorsal line) is sterilized as previously described. A left paracostal incision (7-8 cm) is made through the skin and panniculus, just behind the last rib. The next three intersecting layers of musculature and peritoneum are blunt dissected to create an opening adequate to access the dorsal portion of the rumen.

Bleeding vessels will be clamped and if necessary tied off with absorbable suture.

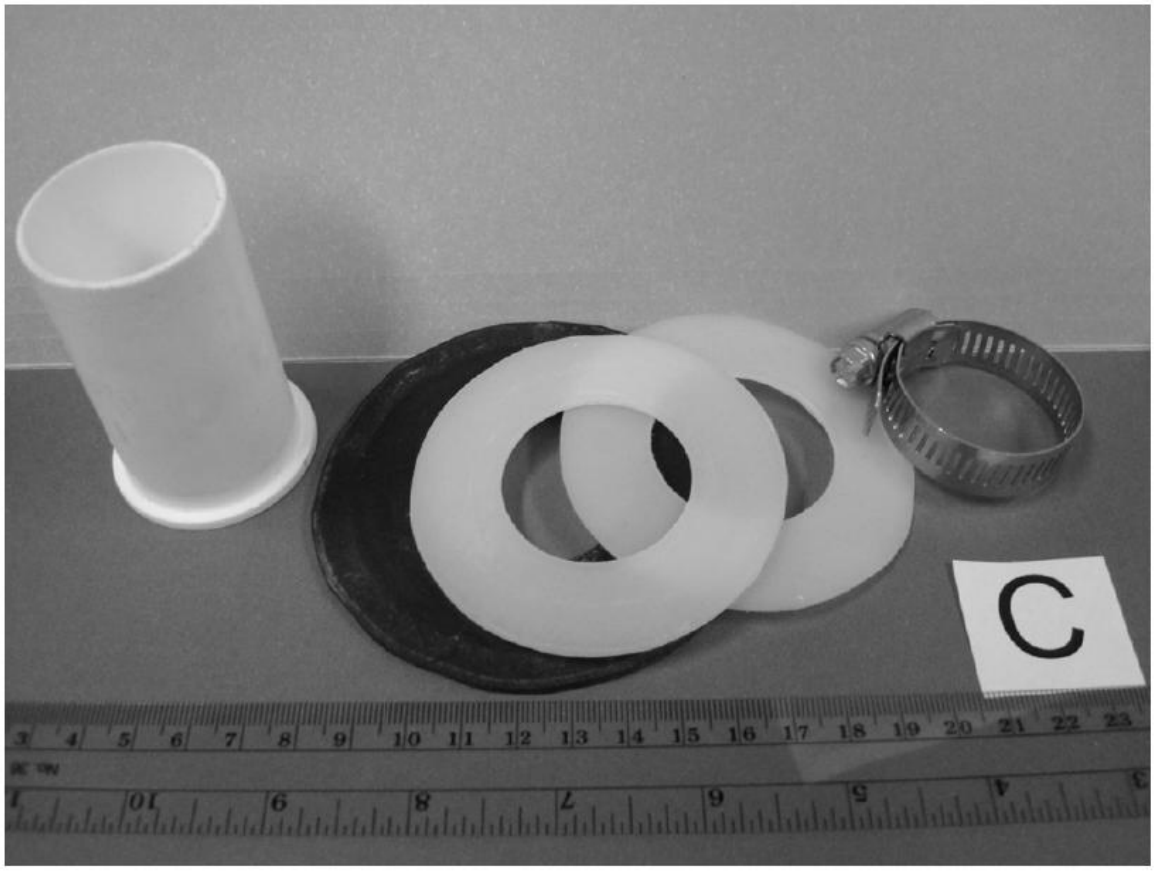
ii. The rumen will be withdrawn through the incision and packed with sterile cotton cloths to avoid spillage of rumen liquor into the abdominal cavity once the rumen wall is incised.

iii. A double purse-string suture pattern (2/0 non-absorbable suture) will be made on the surface of the exposed rumen wall. A 2 cm incision will be made in the center of the purse-string and the flange of the barrel cannula (3" long, barrel diameter 1", flange diameter 2.5") fitted through the opening. The purse-string suture will be delicately drawn snug and lightly knotted to avoid cutting through the rumen tissues. A nylon bullet (3" long) will be attached onto the barrel cannula, a small incision (1 cm) made 2" dorsal to the abdominal incision, and the bullet and cannula pulled through the skin. A cap will replace the bullet and a collar secured around the barrel of the cannula with a stainless steel screw band.

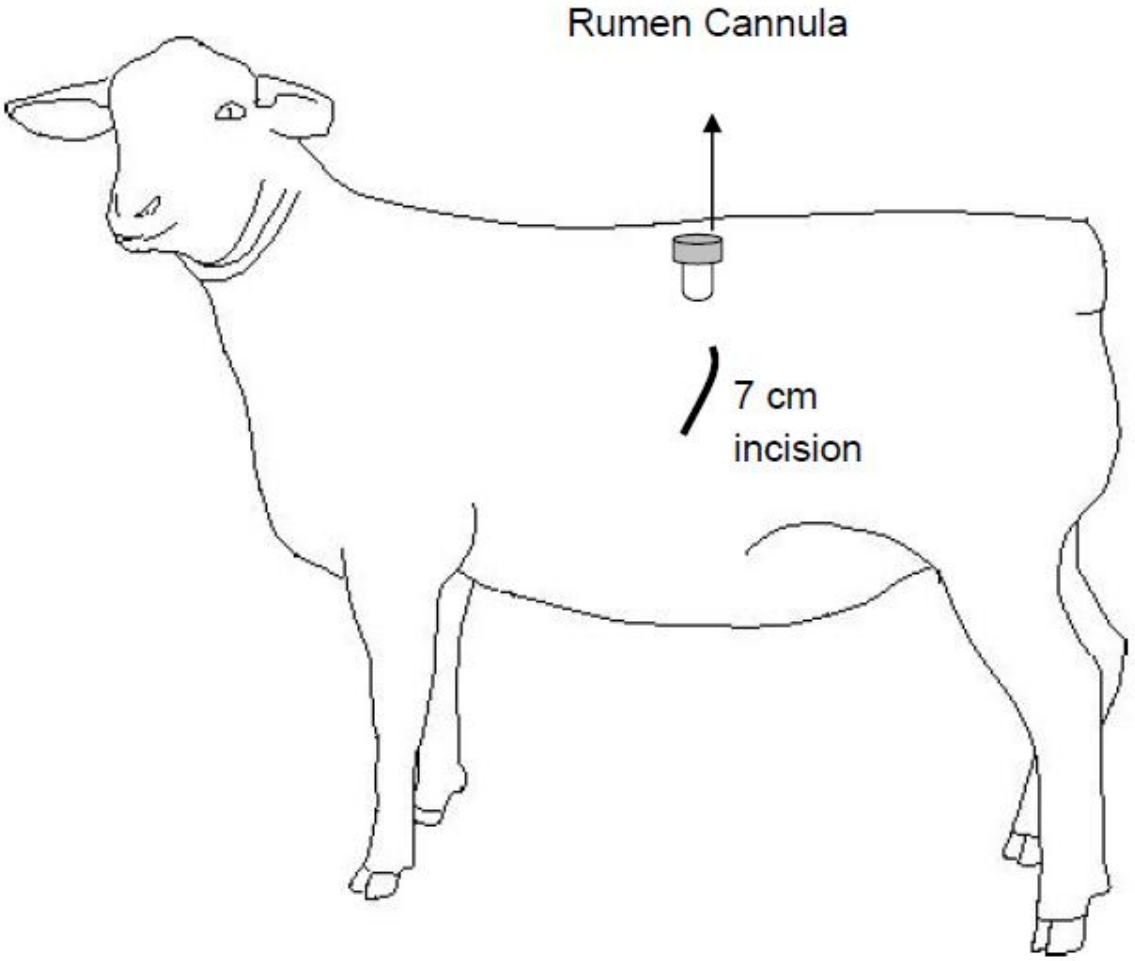
iv. For initial closure, the peritoneum and two innermost layers of abdominal musculature are closed together (continuous pattern) using absorbable suture (2/0 coated vicryl with ½ inch curved-tapered needle). The outermost muscles are then closed separately, again using the above suture. The panniculus ('twitch') muscle is closed separately with absorbable suture (2/0 coated vicryl with ½ inch curved-reverse cutting needle) employing a

continuous pattern. The subcutaneous skin layer is more important in its support than the outer skin layer. To close the subcutaneous layer, use absorbable suture as above, and employ the Surgeon's Continuous Pattern. For the outer skin, use non-absorbable suture (2/0 nylon, Ethilon with 2 inch straight-cutting needle), and close with either a Ford-interlocking or cruciate (horizontal) mattress pattern.

Rumen cannula – barrel-type



Rumen Cannula Incision Site (Right Lateral Recumbency)



2. Rumen biopsy procedure

(IACUC protocol # R-11-33)

1. Sheep will be removed from the metabolic crate during the procedure, and the sheep will be held by personnel to prevent excessive movement. In our experience, after a month of handling and constant contact, the sheep essentially act like pets.
2. The rumen cannula plug will be removed and rumen contents removed by vacuum. The site of biopsy (lateral aspect) will be rinsed of debris with warm saline, and a fiber optic endoscope (Olympus CF, type CF-1T20L and OCV-100) used to visualize the rumen wall.
3. Using a sterilized (70% ethanol) sharpened Allis forceps, rumen epithelial samples (3-5, 10 mg each) will be obtained from the lateral aspect of the rumen. This quantity of tissue is necessary to obtain sufficient RNA and protein for gene and protein expression analysis. On each biopsy occasion, the biopsy sites will be separated by at least 1 inch.
4. Samples will be rinsed and prepared accordingly.
5. The rumen contents will be returned to the rumen, followed by 1 L of warmed rumen buffer solution. The sheep will be returned to the floor pen.
6. Impact of sampling on animal health will be monitored everyday following biopsy for indications of infection (i.e., inflammation, lethargy, anorexia, elevated temperature and food intake).

7. Each biopsy procedure will be separated by at least 3 weeks in any one experiment. Given the high rate of protein turnover of the rumen epithelium (25%/day), the site should heal by at least 6 days.

3. Urea-N kinetics calculations

(based on Lobley et al. (2000))

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

where, E_{D30} and E_{U30} are the enrichments of [$^{15}N^{15}N$]urea in the dose and urine respectively

and, D is the rate of infusion rate of the dose (mass/time).

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

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

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

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

where, $\rho = \frac{UUE_{29}}{UUE_{29}+UUE_{30}}$

and , UUE_{29} and UUE_{30} are the amounts of [$^{14}N^{15}N$] and [$^{15}N^{15}N$] excreted in urine respectively.

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

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

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

Urea-N utilized as absorbed amino acids (a) is calculated indirectly by difference as,

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

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

BIBLIOGRAPHY

Abdoun, K., F. Stumpff, I. Rabbani, and H. Martens. 2010. Modulation of urea transport across sheep rumen epithelium in vitro by SCFA and CO_2. Am. J. Physiol. Gastrointest. Liver Physiol. 298:G190.

Abdoun K., F. Stumpff, K. Wolf, and H. Martens. 2005. Modulation of electroneutral Na transport in sheep rumen epithelium by luminal ammonia. Am. J. Physiol. Gastrointest. Liver Physiol. 289:G508.

Abdul-Razzaq, H. A., and R. Bickerstaffe. 1989. The influence of rumen volatile fatty acids on protein metabolism in growing lambs. Br. J. Nutr. 62:297.

Annison, E., R. Leng, D. Lindsay, and R. White. 1963. The metabolism of acetic acid, propionic acid and butyric acid in sheep. Biochem. J. 88:248.

Archibeque, S., J. Burns, and G. Huntington. 2001. Urea flux in beef steers: Effects of forage species and nitrogen fertilization. J.Anim. Sci. 79:1937.

Baldwin, R. L., and B. Jesse. 1992. Developmental changes in glucose and butyrate metabolism by isolated sheep ruminal cells. J. Nutr. 122:1149.

Baldwin, R. L., R. W. Li, C. Li, J. M. Thomson, and B. J. Bequette. 2012. Characterization of the longissimus lumborum transcriptome response to adding propionate to the diet of growing Angus beef steers. Physiol. Genomics. 44**:**543.

Beever, D. E., and P. T. Doyle. 2007. Feed conversion efficiency as a key determinant of dairy herd performance: A review. Aust. J. Exp. Agric. 47:645.

Bequette, B. J., and N. E. Sunny. 2005. Reducing nitrogen excretion in ruminants: the potential to increase urea recycling. Proceedings of the 3rd Mid-Atlantic Nutrition Conference.

Bergman, E. N., and J. E. Wolff. 1971. Metabolism of volatile fatty acids by liver and portal-drained viscera in sheep. Am. J. Physiol. 221**:**586.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70**:**567.

Bergman, E. N., R. Reid, M. G. Murray, J. Brockway, and F. Whitelaw. 1965. Interconversions and production of volatile fatty acids in the sheep rumen. Biochem. J. 97:53.

Berthelot, V., S. G. Pierzynowski, D. Sauvant, and N. B. Kristensen. 2002. Hepatic metabolism of propionate and methylmalonate in growing lambs. Livest. Prod. Sci. 74:33.

Bradford, E., R. L. Baldwin, H. Blackburn, K. Cassman, P. Crosson, C. Delgado, J. Fadel, H. Fitzhugh, M. Gill, and J. Oltjen. 1999. Animal agriculture and global food supply. Task Force Rep. 135.

Chamberlain, D. G., P. C. Thomas, W. Wilson, C. J. Newbold, and J. C. Macdonald. 1985. The effects of carbohydrate supplements on ruminal concentrations of ammonia in animals given diets of grass silage. J. Agric. Sci. 104:331.

Cohen, N. S., and A. Kuda. 1996. Arginosuccinate synthetase and arginosuccinate lyase are localized around mitochondria: an immunocytochemical study. J. Cell. Biochem. 60:334.

Connell, A., A. Calder, and S. Anderson. 1997. Hepatic protein synthesis in the sheep: effect of intake as by use of stable-isotope-labelled glycine, leucine and phenylalanine. Br. J. Nutr. 77:255.

Danfær, A., V. Tetens, and N. Agergaard. 1995. Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. Comp. Biochem. Physiol. Part B. Biochem. Mol. Biol. 111**:**201.

Demigné, C., C. Yacoub, C. Morand, and C. Rémésy. 1991. Interactions between propionate and amino acid metabolism in isolated sheep hepatocytes. Br. J. Nutr. 65**:**301.

Dijkstra, J., J. M. Forbes, and J. France. 2005. Quantitative aspects of ruminant digestion and metabolism. 2nd ed. CABI, Willingford, UK.

Doranalli, K., G. B. Penner, and T. Mutsvangwa. 2011. Feeding oscillating dietary crude protein concentrations increases nitrogen utilization in growing lambs and this response is partly attributable to increased urea transfer to the rumen. J. Nutr. 141:560.

El-Kadi, S. W., R. L. Baldwin, N. E. Sunny, S. L. Owens, and B. J. Bequette. 2006. Intestinal protein supply alters amino acid, but not glucose, metabolism by the sheep gastrointestinal tract. J. Nutr. 136:1261.

El-Kadi, S. W., A. Suryawan, M.C. Gazzaneo, N. Srivastava, R. A. Orellana, H. V. Nguyen, G. E. Lobley, and T. A. Davis. 2012. Anabolic signaling and protein deposition are enhanced by intermittent compared with continuous feeding in skeletal muscle of neonates. Am. J. Physiol. Endocrinol. Metab. 302:E674.

Emmanuel, B. 1980. Oxidation of butyrate to ketone bodies and CO_2 in the rumen epithelium, liver, kidney, heart and lung of camel (Camelus dromedarius), sheep (Ovis aries) and goat (Carpa hircus). Comp. Biochem. Physiol. 65 B:699.

Federal Register. 2001. Clean Water Act 33 U.S.C. 1311, 1314, 1316, 1317, 1318, 1342 and 1361: Proposed Rules. 66:9.

Fernandez, C. A., C. Des Rosiers, S. F. Previs, F. David, and H. Brunengraber. 1996. Correction of ^{13}C mass isotopomer distributions for natural stable isotope abundance. J. Mass Spectrom. 31**:**255.

Fluharty, F., K. McClure, M. Solomon, D. Clevenger, and G. Lowe. 1999. Energy source and ionophore supplementation effects on lamb growth, carcass characteristics, visceral organ mass, diet digestibility, and nitrogen metabolism. J. Anim. Sci. 77**:**816.

Fujita, T., M. Kajita, and H. Sano. 2006. Responses of whole body protein synthesis, nitrogen retention and glucose kinetics to supplemental starch in goats. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 144**:**180.

Górka P., Z. M. Kowalski, P. Pietrzak, A. Kotunia, W. Jagusiak, J. J. Horst, P. Guilloteau, and R. Zabielski. 2011. Effect of method of delivery of sodium butyrate on rumen development in newborn calves. J. Dairy Sci. 94:5578.

Gozho, G., M. Hobin, and T. Mutsvangwa. 2008. Interactions between barley grain processing and source of supplemental dietary fat on nitrogen metabolism and urea-nitrogen recycling in dairy cows. J. Dairy Sci. 91:247.

Hachey, D. L., W. R. Parsons, S. McKay, and M. W. Haymond. 1999. Quantitation of monosaccharide isotopic enrichment in physiologic fluids by electron ionization or negative chemical ionization GC/MS using di-O-isopropylidene derivatives. Anal. Chem. 71**:**4734.

Haüssinger, D. 1990. Nitrogen metabolism in liver: structural and functional organization and physiological relevance. Biochem. J. 267:281.

Huntington, G. B., and S. Archibeque. 1999. Practical aspects of urea and ammonia metabolism in ruminants. Proc. Am. Soc. Anim. Sci.

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.

Huntington, G. B., K. Magee, A. Matthews, M. Poore, and J. Burns. 2009. Urea metabolism in beef steers fed tall fescue, orchardgrass, or gamagrass hays. J. Anim. Sci. 87:1346.

Jackson, A. A., D. Picou, and J. Landman. 1984. The non-invasive measurement of urea kinetics in normal man by a constant infusion of $^{15}N^{15}N$-urea. Human Nutr. Clin. Nutr. 38C:339.

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.

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

Kim, H., J. Choung, D. Chamberlain, and G. E. Lobley. 1999. Effect of propionate on ovine urea kinetics. Page 57 in Protein Metabolism and Nutrition. G. E. Lobley, A. White, and J. C. McCrae, ed. Eur. Assoc. Anim. Prod., Wageningen Press, Wageningen, UK.

King, K. R., J. M. Gooden, and E. F. Annison. 1985. Acetate metabolism in the mammary gland of the lactating ewe. Aust. J. Biol. Sci. 38:23.

Krehbiel, C., D. Harmon, and J. Schneider. 1992. Effect of increasing ruminal butyrate on portal and hepatic nutrient flux in steers. J. Anim. Sci. 70:904.

Kristensen, N. B., and D. L. Harmon. 2004.Splanchnic metabolism of volatile fatty acids absorbed from the washed reticulorumen of steers. J. Anim. Sci. 82:2033.

Lapierre, H. and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: a review. J. Dairy Sci. 84:E223.

Li, R. W., S. Wu, W. Li, and C. Li. 2012. Perturbation dynamics of the rumen microbiota in response to exogenous butyrate. PloS one. 7:e29392.

Lobley, G. E. and G. Milano. 1997. Regulation of hepatic nitrogen metabolism in ruminants. Proc. Nutr. Soc. 46:547.

Lobley, G. E., D. M. Bremmer, and G. Zuur. 2000. Effects of diet quality on urea fates in sheep as assessed by refined, non-invasive [^{15}N-^{15}N] urea kinetics. Br. J. Nutr. 84:459.

Loosli, J. K., and I. W. McDonald. 1968. Nonprotein nitrogen in nutrition of ruminants. FAO Agricultural Studies No. 73.

Ludden, P. A, R. M. Stohrer, K. J. Austin, R. L. Atkinson, E. L. Belden, and H. J. Harlow. 2009. Effect of protein supplementation on expression and distribution of urea transporter-B in lambs fed low-quality forage. J. Anim. Sci. 87:1354.

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.

MacRae, J. C., L. A. Bruce, D. S. Brown, D. Farningham, and M. Franklin. 1997. 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.

MacRae, J. C., L. A. Bruce, and D. S. Brown. 1995. Efficiency of utilization of absorbed amino acids in growing lambs given forage and forage: barley diets. Anim. Sci. 61:277.

Marini, J., and M. Van Amburgh. 2003. Nitrogen metabolism and recycling in Holstein heifers. J. Anim. Sci. 81:545.

Marini, J., J. Klein, J. Sands, and. M. Van Amburgh. 2004. Effect of nitrogen intake on nitrogen recycling and urea transporter abundance in lambs. J. Anim. Sci. 82:1157.

Moloney, A. 1998. Growth and carcass composition in sheep offered iso-energetic rations which resulted in different concentrations of ruminal metabolites. Livest. Prod. Sci. 56**:**157.

Mosenthin, R., W. Sauer, and C. de Lange. 1992. Tracer studies of urea kinetics in growing pigs: I. The effect of intravenous infusion of urea on urea recycling and the site of urea secretion into the gastrointestinal tract. J. Anim. Sci. 70:3458.

Norton, B., A. Janes, and D. Armstrong. 1982. The effects of intraruminal infusions of sodium bicarbonate, ammonium chloride and sodium butyrate on urea metabolism in sheep. Br. J. Nutr. 48:265.

NRC. 2007. Nutrient requirements of small ruminants: Sheep, Goats, Cervids, and new world Camelids. National Academy Press, Washington, DC.

Oba, M., R. L. Baldwin, S. L. Owens, and B. J. Bequette. 2004. Urea synthesis by ruminal epithelial and duodenal mucosal cells from growing sheep. J. Dairy Sci. 87**:**1803.

Obara, Y., and D. W. Dellow. 1994. Influence of energy supplementation on nitrogen kinetics in the rumen and urea metabolism. Japan Agric. Res. Q. 28**:**143.

Obara, Y., Y. Sasaki, S. Watanabe, and T. Tsuda. 1971. The effects of vagatomy on hyperglycemia by ruminal administration of butyric acid in sheep. Tohoku J. Agric. Res. 22:156.

Ørskov, E. R. 1986. Starch digestion and utilization in ruminants. J. Anim. Sci. 63:1624.

Ørskov, E. R., N. A. MacLeod, and Y. Nakashima. 1991. Effect of different volatile fatty acids mixtures on energy metabolism in cattle. J. Anim. Sci. 69:3389.

Ortigues-Marty, I. 2003. Effects of intraruminal propionate supplementation on nitrogen utilisation by the portal-drained viscera, the liver and the hindlimb in lambs fed frozen rye grass. Br. J. Nutr. 90**:**939.

Rémond D., J. P. Chaise, E. Delval, C. Poncet. 1993. Net transfer of urea and ammonia across the ruminal wall of sheep. J. Anim. Sci. 71:2785.

Rémond, D., L. Bernard, I. Savary-Auzeloux, and P. Nozière. 2009. Partitioning of nutrient net fluxes across the portal-drained viscera in sheep fed twice daily: effect of dietary protein degradability. Br. J. Nutr. 102:370.

Rémond, D., F. Meschy, and R. Boivin. 1996. Metabolites, water and mineral exchanges across the rumen wall: mechanisms and regulation. Ann. Zootech. 45:97.

Reynolds, C. K., and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis. J. Anim. Sci. 86:E293.

Reynolds, C. K. 1992. Metabolism of nitrogenous compounds by ruminant liver. J. Nutr. 122:850.

Ritzhaupt, A., I. Wood, A. Jackson, B. Moran, and S. 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:S122.

Røjen, B. A., S. B. Poulsen, P. K. Theil, R. A. Fenton, and N. B. Kristensen. 2011. Short communication: Effects of dietary nitrogen concentration on messenger RNA expression and protein abundance of urea transporter-B and aquaporins in ruminal papillae from lactating Holstein cows. J. Dairy Sci. 94:2587.

Russell, K., G. E. Lobley, J. Rawlings, D. J. Millward and E. J. Harper. 2000. Urea kinetics of a carnivore, Felis silvestris catus. Br. J. Nutr. 84:597.

Sakata, T., and H. Tamate. 1978. Rumen epithelial cell proliferation accelerated by rapid increase in intraruminal butyrate. J. Dairy Sci. 61:1109.

Sakata, T., and H. Tamate. 1979. Rumen epithelium cell proliferation accelerated by propionate and acetate. J. Dairy Sci. 62**:**49.

Sano, H., N. Hattori, Y. Todome, J. Tsuroka, H. Tukashashi, and Y. Terashima. 1993. Plasma insulin and glucagon responses to intravenous infusion of propionate and their autonomic control in sheep. J. Anim. Sci. 71:3414.

Santos, F. A. P., J. E. P. Santos, C. B. Theurer, and J. T. Huber. 1998. Effects of rumen-undegradable protein on dairy cow performance: A 12-year literature review. J. Dairy Sci. 81:3182.

Sarraseca, A., E. Milne, M. Metcalf, and G. E. Lobley. 1998. Urea recycling in sheep: effects of intake. Br. J. Nutr. 79:79.

Seal, C., and D. Parker. 1996. Effect of intraruminal propionic acid infusion on metabolism of mesenteric-and portal-drained viscera in growing steers fed a forage diet: II. Ammonia, urea, amino acids, and peptides. J. Anim. Sci. 74**:**245.

Shen, Z., H. M. Seyfert, B. Löhrke, F. Schneider, R. Zitnan, A. Chudy, S. Kuhla, H. M. Hammon, J. W. Blum, and H. Martens. 2004. An energy-rich diet causes rumen papillae proliferation associated with more IGF type 1 receptors and increased plasma IGF-1 concentrations in young goats. J. Nutr. 134:11.

Simmons, N., A. Chaudhry, C. Graham, E. Scriven, A. Thistlethwaite, C. Smith, and G. Stewart. 2009. Dietary regulation of ruminal bovine UT-B urea transporter expression and localization. J. Anim. Sci. 87:3288.

Speedy, A. W. Global production and consumption of animal source foods. 2003. J. Nutr. 133:4048S.

Stern, M. D., and W. H. Hoover. 1979. Methods for determining and factors affecting rumen microbial protein synthesis: a review. J. Anim. Sci. 49**:**1590.

Stewart, G. S., C. Graham, S. Cattell, T. P. L. Smith, N. L. Simmons, and C. P. Smith. 2005. UT-B is expressed in bovine rumen: potential role in ruminal urea transport. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289:R605.

Stewart, P. M., and M. Walser. 1980. Failure of the normal ureagenic response to amino acids in organic acid-loaded rats: proposed mechanism for the hyperammonemia of propionic and methylmalonic acidemia. J. Clin. Investig. 66**:**484.

Storm, A. C., M. Hanigan, and N. B. Kristensen. 2011. Effects of ruminal ammonia and butyrate concentrations on reticuloruminal epithelial blood flow and volatile fatty acid absorption kinetics under washed reticulorumen conditions in lactating dairy cows. J. Dairy Sci. 94:3980.

Storm, E., and E. Orskov. 1983. The nutritive value of rumen micro-organisms in ruminants. 1. Large-scale isolation and chemical composition of rumen micro-organisms. Br. J. Nutr. 50:463.

Sun, Y. B., and G. Y. Zhao. 2009. The relationship between the volatile fatty acids supply and the nitrogen retention in growing sheep nourished by total intragastric infusions. Sm. Rumin. Res. 81**:**8.

Sunny, N. E., S. L. Owens, R. L. Baldwin, S. W. El-Kadi, R. A. Kohn, and B. J. Bequette. 2007. Salvage of blood urea nitrogen in sheep is highly dependent on plasma urea concentration and the efficiency of capture within the digestive tract. J. Anim. Sci. 85:1006.

Tayek, J. A. & Katz, J. 1997. Glucose production, recycling, Cori cycle, and gluconeogenesis in humans: relationship to serum cortisol. Am. J. Physiol. 272**:** E476.

UN. 2012. World population prospects: the 2012 revision. United Nations, ESA/P/WP.227.

Walpole, M. E., B. L. Schurmann, P. Górka, G. B. Penner, M. E. Loewen, and T. Mutsvangwa. 2013. Functional role of aquaporins and urea transporters in urea flux across the ruminal epithelium. Page 269 in Energy and protein metabolism and nutrition in sustainable animal production. J. W. Oltjen, E. Kebreab, H. Lapierre, ed. EAAP publication no. 134.

Wickersham, T., E. Titgemeyer, and R. Cochran. 2008. Methodology for concurrent determination of urea kinetics and the capture of recycled urea nitrogen by ruminal microbes in cattle. Animal. 3:372.

Wu, G. 1995. Urea synthesis in enterocytes of developing pigs. Biochem. J. 312:717.