ABSTRACT

Title of dissertation: UREA-N RECYCLING IN LACTATING DAIRY COWS

Sarah Katherine Dinh, Doctor of Philosophy, 2007

Dissertation directed by: Professor Richard A. Kohn
Department of Animal and Avian Sciences

This study was designed to determine the effect of rumen degradable protein (RDP) and rumen undegradable protein (RUP) on urea-N recycling and microbial N flow. Eight mid-lactation Holstein cows were assigned to a repeated 4 x 4 Latin square. The diets were isoenergetic with RDP and RUP concentrations arranged in a factorial design (10.0 and 12.5% RDP and 5.6 and 8.1% RUP as a percentage of dry matter).

The 10.0% RDP diets resulted in greater milk yield and lower milk protein concentration than the 12.5% RDP diets. High RUP diets tended ($P = 0.1$) to increase bacterial N flow in the liquid fraction. The NRC 1989 model predicted flow of microbial N and total N from the rumen more accurately than the NRC 2001. The NRC 2001 model predicted a higher RDP requirement and a lower RUP requirement for all four diets compared with the NRC 1989 model. Both models reflect the dietary changes that were intended by increasing the RDP and RUP in a factorial manner. There was no effect of RUP or RDP on the g/d of urea-N transferred from the blood to the gut or returning to the ornithine-urea cycle. However, plasma urea-N (PUN) incorporated into rumen microbial protein tended ($P = 0.14$) to increase with the low RDP diets. Rate of transfer of PUN to the gut appeared to be independent of PUN concentrations. Expression of urea transporter (bUT-B2) gene in the rumen epithelial did not change due to diet. As dietary protein

intake increased, a constant amount (g/d), or decreasing fraction (g/g), of PUN was transferred to the gut. The apparent saturation of urea transporters in the gut prevented excess PUN from recycling to the gut on high-protein diets. Thus, the energy costs for N excretion is less than that which would have resulted if a constant percentage of PUN recycled.

UREA-N RECYCLING IN LACTATING DAIRY COWS

By

Sarah Katherine Dinh

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland-College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

Advisory Committee:

Professor Richard A. Kohn, Chair
Dr. Ransom L. Baldwin, VI
Assistant Professor Brian J. Bequette
Professor Thomas W. Castonguay
Professor Richard A. Erdman

ACKNOWLEDGEMENTS

I would like to thank Dr. Richard Kohn for serving as my advisor for my Ph.D. career. I am grateful for the constant encouragement to get over that next hurdle and because of that I have a lot more confidence in what I can accomplish.

Thank you to Dr. Randy Baldwin for all your thoughts on how to get the most out of this project and for wrangling some unruly cows. Thank you, Dennis, for always being there to help take samples and make my life a little easier. Mary, you made analyzing all those tissues samples look easy. Thank you for all your time and effort. Thanks to Dr. Castonguay, Dr. Erdman, and Dr. Bequette for taking time out of your very busy schedules to serve on my committee.

I also need to thank Telmo Oleas and Emilio Ungerfield for giving of their time to help me take samples and figure out lab procedures. You both made analyzing all my samples so much easier than I thought it could be. And a thank you goes out to Sarah, Melissa, Lynn, and Jessica for volunteering to take samples in the middle of the night so I could get some much needed rest. Thank you to Kyo for spending so much time helping analyze all the samples. Thank you to Robert for working so hard taking and analyzing samples, and always looking for ways to improve procedures. To Ashley and Juen thanks for all the advice as to how to get through a Ph.D. in one piece.

To my parents, thank you for all your love and support. You have always been there for me, and bring so much stability to my life, which has been priceless during the craziness of the past few years. To my brother Jake and his wife Jennifer, thank you for all your support and providing a break from the stresses of graduate school.

A special thank you goes out to my husband, Binh. You are always there whenever I need a laugh or a shoulder to cry on. I'm not sure how I would have gotten through the last few years without you. You are my love and my inspiration.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a..... fraction of GER used for anabolic purposes

ADF..... acid detergent fiber

AP .....absorbed protein

APE..... atom percent excess

BW .....body weight

CP..... crude protein

CR ..... clearance rate

DIM.....days in milk

DM .....dry matter

DMI..... dry matter intake

f .....fraction of GER that went to feces

FCM .....fat corrected milk

GER.....GIT entry rate

GIT..... gastrointestinal tract

IMCD ..... inner medullary collecting duct

LAB..... liquid associated bacteria

MN .....microbial nitrogen

MNU ..... microbial nitrogen derived from recycled blood urea nitrogen

MP.....metabolizable protein

MUN .....milk urea nitrogen

N..... nitrogen

NDF..... neutral detergent fiber

NE_L .....net energy of lactation

PAN.....plasma ammonia nitrogen

PCR.....polymerase chain reaction

PDV.....portal drained viscera

PUN.....plasma urea nitrogen

r .....fraction of GER returning to ornithine-urea cycle

RAN .....rumen ammonia nitrogen

RAP.....rumen available protein

RDP..... rumen degradable protein

RMSPE .....root mean square prediction error

ROC ..... urea-N return to ornithine-urea cycle from GIT

RUP..... rumen undegradable protein

SAB.....solids associated bacteria

SEM ..... standard error of the mean

TMR.....total mixed ration

U.....proportion of dose eliminated in urine

UER.....urea-N entry rate into blood from liver

UFE.....urea-N fecal excretion

UUA.....urea-N utilized for anabolic purposes

UUE .....urinary urea-N elimination

UUN.....urinary urea nitrogen

VFA.....volatile fatty acids

CHAPTER 1

REVIEW OF LITERATURE

INTRODUCTION

Improving nitrogen (N) utilization by dairy cows can reduce feed costs and losses of N to the environment. If the same amount of milk can be produced on a diet that is lower in protein, feed costs may be decreased because protein is one of the most expensive components of the diet. In terms of reducing N losses to the environment, feeding cows less protein decreases N excretion in urine. Urea in urine is quickly converted to ammonia (NH_3) by microbial ureases (present in feces), and ammonia readily volatilizes when manure is stored or applied to crops. The most effective way to decrease N losses from dairy farms is through improvement of conversion efficiency of feed N to animal product (Kohn et al., 1997). However, feeding dairy cows more efficiently requires that we accurately estimate the N requirements of the cow, and this requires an improvement in our current understanding of urea-N recycling.

Nitrogen recycling is an important process in ruminants and has been the subject of previous research, but it remains unclear how N recycling affects the N requirement of the lactating cow. Much of feed protein is broken down in the rumen producing ammonia, which subsequently can be used by rumen microbes to synthesize microbial protein. Ammonia is also absorbed from the gut and detoxified in the liver to urea through the ornithine-urea cycle and released into the blood. Blood urea can either be excreted in the urine or transferred to the digestive tract. Urea-N transferred to the digestive tract is largely broken down to ammonia and utilized as a source of N for microbial protein synthesis. Because the microbial protein synthesized from the recycled N in the rumen can be digested and absorbed as a protein source for the animal, urea-N transferred to the rumen has greater potential to improve the efficiency of N utilization of

ruminants compared with urea-N transferred to the lower gastrointestinal tract. The same cannot be said for microbial protein synthesized in the lower tract.

Dietary factors can affecting urea-N recycling include, amount of protein in the diet (Marini and Van Amburgh, 2003), dry matter intake (Sarraseca et al., 1998), forage-to-concentrate ratio (Huntington et al., 1996), and diet digestibility (Theurer et al., 2002). All have been shown to affect, in part, both the amount of ammonia absorbed from the rumen and PUN transfer from blood to gastrointestinal tract. Likewise, ammonia concentration in the rumen can affect how much urea-N is transferred to the rumen for use by microbes (Cheng and Wallace, 1979 and Kennedy and Milligan, 1978).

Renal function may affect efficiency of N utilization in the cow and urea-N recycling. Because kidneys salvage urea-N when dietary protein concentration or intake are low and facilitates excretion of excess urea-N when dietary protein concentration or intake are high (Levinsky et al., 1959, Marini et al., 2004, Marini and Van Amburgh, 2003). Regulation of the kidney function dampens changes in blood urea concentrations that occur due to different diets. Ultimately, this affects the urea-N available for transfer to the rumen. Even though the kidney plays a major role in maintaining blood urea-N concentrations, its impact on urea-N recycling to the rumen has not been quantified in dairy cows.

Little published data exists that quantify urea-N recycling in lactating dairy cows, therefore, the aim of this study was to quantify urea-N recycling and factors that influence and control it. The first objective was to determine quantitatively how RDP and RUP affect N recycling as well as rumen microbial protein synthesis. The second objective was to evaluate means of control of N recycling to the rumen as affected by regulation of urea transporters in the rumen wall, uptake of ammonia by rumen microbes,

and regulation of excretion at the kidney. Lastly, the NRC 1989 and 2001 were evaluated to assess how well they predict N flux from the rumen and ruminal N balance.

Ammonia Absorption

Ammonia resulting from microbial breakdown of dietary protein is either incorporated into microbial protein or absorbed across the wall of the digestive tract into the blood. Reynolds and Huntington (1988) determined that the lower gastrointestinal tract (GIT) including the small intestines, large intestines, and cecum, accounted for only 33% of ammonia absorption across the whole GIT illustrating the importance of the rumen as the major site of ammonia absorption. Diet type has a greater influence on ammonia absorption from the rumen compared to lower GIT (Huntington, 1986; Reynolds and Huntington, 1988). In fact, absorption of ammonia from the whole GIT decreased when high concentrate diets were fed compared with forage diets, while ammonia absorption from the lower GIT was unaffected (Huntington, 1986; Reynolds and Huntington, 1988). Although much of the ammonia produced in the rumen that is available for absorption is absorbed into the blood as NH_4^+ there is evidence that diets resulting in increased rumen ammonia N (RAN) concentration also exhibited increased rumen wall metabolism of ammonia (Nocek et al., 1980).

Ammonia-N accounts for 71% of dietary protein degradation and it is estimated that 45% of plasma urea-N is derived from RAN (Nolan and Leng, 1972). In the rumen, ammonia exits as two forms, the non-ionized form (NH_3) is freely diffusable across the lipid bilayers of the rumen wall, whereas the ionized form (NH_4^+) is not (Hogan, 1961). A recent review by Abdoun et al. (2006) suggested the possibility of NH_4^+ transport proteins in the rumen wall, but currently it is assumed that NH_4^+ is transported into the blood via potassium channels. Regardless, the ruminal concentration of NH_3 explains

more of the variation in ammonia-N absorption compared with total ammonia concentration (NH_3 plus NH_4^+; Siddons et al., 1985). The main determinant of the ratio of NH_3: NH_4^+ in the rumen fluid is pH. As the rumen pH increases there is an increase in the NH_3: NH_4^+ due to the pKa of 9.3. Therefore, dietary alterations that alter pH will also affect ammonia absorption and potentially urea-N recycling.

Ornithine-Urea Cycle

The ornithine-urea cycle (Figure 1-1) is the only pathway for NH_3 detoxification to produce urea and occurs mainly in the periportal cells of the liver. However, there is evidence that urea synthesis can also occur in rumen epithelial and duodenal mucosal cells (Oba et al., 2004). The ornithine cycle, as reviewed by Garret and Grisham (1999), proceeds from the initial step where carbamoyl-phophate reacts with ornithine in the mitochondria to form citrulline through the action of ornithine transcarbamoylase. Carbamoyl-P formation requires ATP, and is formed from one bicarbonate ion and one ammonia molecule, which provides one of the N for urea synthesis. Wolf (1981) demonstrated that the carbon in urea, supplied from the bicarbonate ion, is selectively reincorporated into urea. Citrulline is transported out of the mitochondria to the cytosol where, with the input of aspartate, argininosuccinate is formed by argininosuccinate synthetase in an additional ATP dependent reaction. The addition of N from aspartate at this step provides the second N for the urea formed. Subsequently, argininosuccinate lyase removes fumarate, which can enter the tricarboxylic acid cycle and serve as an intermediate for aspartate production, leaving arginine. Arginase completes the ornithine cycle by cleaving arginine to ornithine and urea.

The process of converting ammonia to urea requires energy input of 3 ATP and conversion of one aspartate molecule to fumarate. Lobley et al. (1995) observed that

with varied ammonia infusion rates, the maximum proportion of urea-N, which could be derived from NH_3, ranged from 0.66 and 0.54. At lower infusion rates the shortfall in N supply for urea synthesis could be augmented by hepatic uptake of free amino acids. At the higher infusion rate other substrates would be used to make up for the shortfall in N for urea synthesis, which could negatively impact the N economy of the

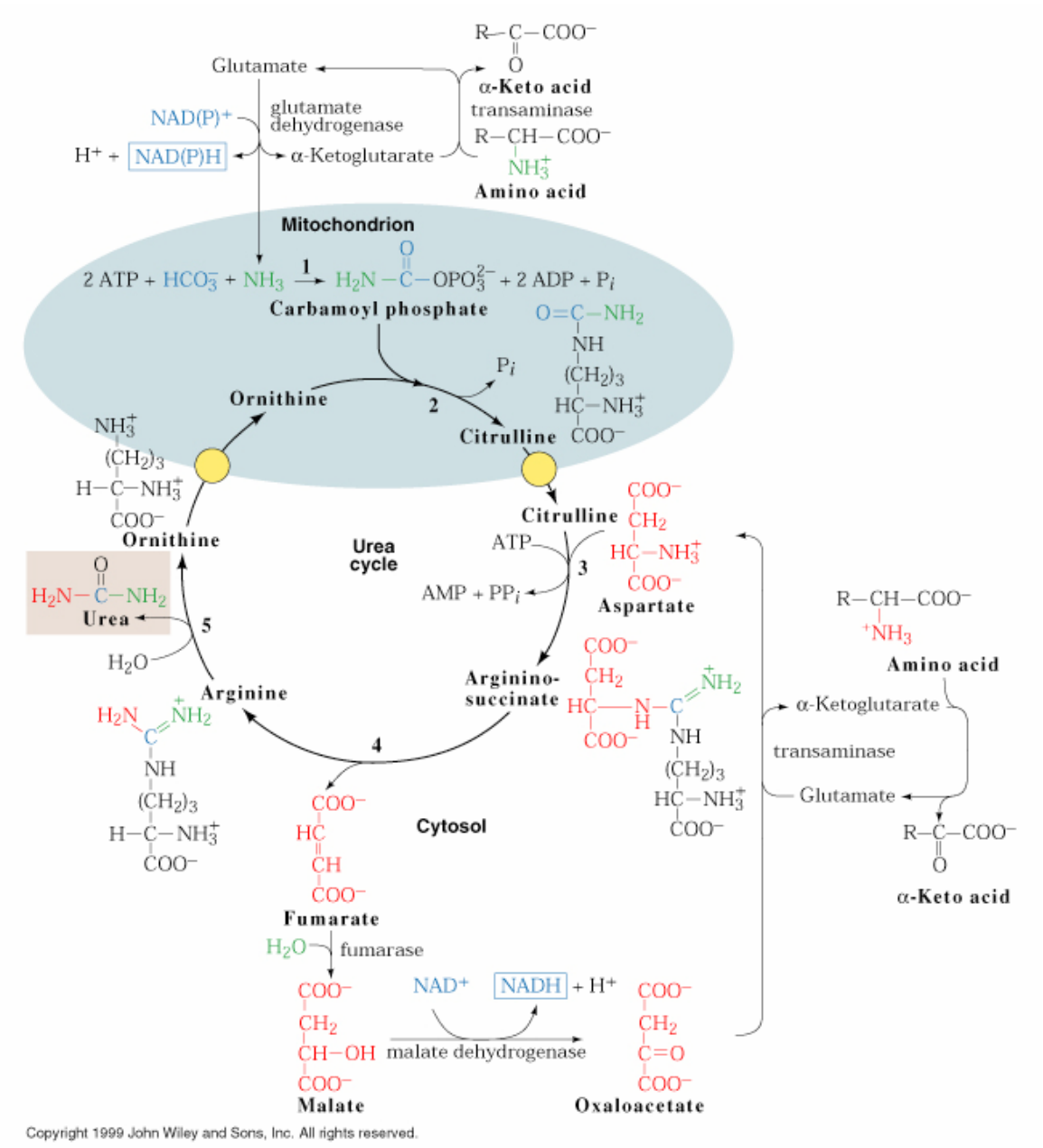


Figure 1. Ornithine-urea cycle and its inputs

animal. However, Moorby and Theobald (1999) infused ammonium acetate into the duodenum of lactating dairy cows and found no significant difference in the N balance between the cows that were infused and the control cows indicating that urea synthesis had no additional negative effect on amino acid availability for milk production, and demonstrating amino acids were not limiting. However, they conceded that N absorption, in the form of ammonia and conversion to urea could represent a loss of protein to the cow due to the excretion of urea-N that could have served as a N source for microbial production if it had been transferred to the rumen.

It is unclear whether urea-N recycling negatively impacts the N economy of the cow by decreasing amino acid availability for milk production, but it is possible for both N atoms in urea to derive from absorbed NH_3. If the latter occurred urea-N recycling would not negatively impact the N economy. In the most commonly occurring version of ornithine-urea cycle, carbamoyl phophate synthetase incorporates NH_3 to form carbamoyl-P, which along with ornithine is used to produce citrulline. Alternatively, glutamate dehydrogenase can incorporate NH_4^+ to form glutamate, with glutamate transaminating to form aspartate by action of glutamate-aspartate aminotransferase. In this case, the N supplied by aspartate originates from NH_4^+ with no net penalty on amino acid availability. However, Lobley et al. (1995) and Lobley et al. (1996) reported that infusion of ^{15}N labeled ammonium chloride into the mesenteric vein resulted in formation of [^{15}N]glutamate, but the ratio of double-labeled urea ([$^{15}N^{15}N$]urea) to single-labeled urea ([$^{15}N^{14}N$]urea) was 97:3 indicating little formation of double-labeled urea from amino acids, which should have occurred if the glutamate was then converted to aspartate. Their explanation was that the site of glutamate uptake and synthesis was downstream from the site of ureagenesis inhibiting formation of double-labeled urea from

amino acids. If both N atoms in the urea molecule could be provided by ammonia the need for amino acids to provide the additional N needed to produce urea would be eliminated.

Although one of the main products of the ornithine-urea cycle is urea, there is evidence that the main role of the ornithine-urea cycle is to detoxify ammonia, not to produce urea (Meijer et al., 1985). When ornithine was omitted as a substrate in rat hepatocytes there was only a slight inhibition of the urea synthesis at lower amino acid concentrations, and subjecting the hepatocytes to an ornithine cycle inhibitor elicited no effect on rate of urea synthesis indicating that the level of ornithine-urea cycle intermediates, or the enzymes involved, exert little control over ureagenesis (Meijer et al., 1985). When the amino acid concentration was held constant the activation of carbamoyl-phosphate synthetase led to a decrease in the ammonia concentration with only a slight increase in urea synthesis indicating the main purpose of the ornithine cycle is to decrease the ammonia concentration not increase the urea concentration (Meijer et al., 1985). Since urea is a waste product to most animals it follows that producing urea would be an inefficient use of resources.

Urea-N Entry Into the Rumen

The rate of transfer of plasma urea N (PUN) to the GIT in dairy cows is substantial (171 g N/d), accounting for approximately 65% of urea synthesized (Lapierre and Lobley, 2001). Urea-N enters the rumen via either the rumen wall (diffusion or transporter mediated) or with the saliva. The amount entering through saliva depends on the amount of saliva produced and the PUN concentration. Diet impacts how much urea-N enters the rumen through each route with higher forage diets leading to more urea-N entering the rumen through saliva due to greater saliva production from increased

rumination (Huntington, 1989). Similarly, when steers were fed a diet containing 77% steam-flaked or dry-rolled sorghum, both highly digestible in the rumen, the majority of the urea-N entered the rumen through the rumen wall (32.3 and 42.3 g/d for dry-rolled and steam-flaked sorghum, respectively) as opposed to the salivary route (13.3 and 2.0 g/d for the dry-rolled and steam-flaked sorghum, respectively; Theurer et al., 2002).

Urease activity: Urea that diffuses across the rumen wall is quickly converted to ammonia and CO_2 by urease activity of rumen bacteria attached to the rumen wall. Urease activity is reduced when ruminal ammonia concentrations are elevated (Cheng and Wallace, 1979), inhibiting the transfer of PUN into the rumen (Kennedy and Milligan, 1978). More recent work by Marini et al. (2004) support these finding as they observed a linear decrease in urease activity with increasing levels of N intake, which could be attributed to the increase in degradation of feed N leading to an increase in the ammonia concentration. The reducing environment of the rumen, and the pKa of NH_3 (9.3), quickly led to the conversion of NH_3 to NH_4^+. Since NH_4^+ is not diffusible across the rumen wall, it is "trapped" in the rumen, and there is the potential for high ruminal NH_4^+ concentration to inhibit urease activity. Due to its potential inhibitory effect on urea-N entry, it has been shown in cattle that a ruminal ammonia concentration of 50 to 80 mg N/L was associated with maximal transfer of urea-N into the rumen (Kennedy and Milligan, 1978). In contrast, Bunting et al. (1989b) reported that higher RAN concentration only slightly inhibited urease activity.

Dry matter intake: Dry matter intake can also affect urea-N entry into the rumen. Sarraseca et al. (1998) fed sheep at 0.6, 1.2, and 1.8 times maintenance energy requirements (NRC, 1989) and found that as intake increased the amount of urea-N entering the GIT from the blood increased. The proportion of urea-N that entered the

GIT, which was returned to the ornithine-urea cycle, was not affected by intake level (Sarraseca et al., 1998). At higher intake rates of urea-N flux to the rumen increased, but that did not result in increased use for anabolic purposes by the microbes. However, interpretation is difficult because of the confounding factor as intake increased, N intake increased.

Protein intake: Although there is consistency in the literature that feeding a higher protein diet increases PUN concentration and urinary urea-N excretion (Bunting et al., 1989a, Bunting et al., 1987, Marini et al., 2004, Marini and Van Amburgh, 2003, Archibeque et al., 2002), there are conflicting results with regard to the impact of dietary N concentration on urea-N recycling. One study found no effect of dietary N on N recycled to the GIT (Marini and Van Amburgh, 2003), while other work, from the same author, has demonstrated a linear increase in grams of N per day recycled to the GIT as the N content of the diet increased (Marini et al., 2004). Studies using heifer calves (Bunting et al., 1989a) and lambs (Bunting et al., 1987) have demonstrated higher protein diets exhibited decreased abomasal N flow as a percentage of N intake (124% and 71% for a low protein and high protein diet, respectively; Bunting et al., 1989a). This observation could be explained if calves on the low-protein diet recycled more urea-N to the rumen for use by the rumen microbes. Moreover, the percentage of bacterial N derived from PUN was greater for animals fed a low protein diet compared with a high protein diet (Bunting et al., 1989a). Archibeque et al. (2001) compared gama grass with switch grass at two rates of N fertilization and observed urea-N entering the GIT as a percentage of total urea production was 11.46 percentage units lower when steers were fed forage grown with high N fertilization rates and the recycling to the ornithine-urea cycle was increased by 2.17 percentage units. However, as a percentage of urea-N that

entered the GIT there was no difference in recycling of N back to the ornithine-urea cycle. This result illustrates that the diets were delivering N in excess of the steers needs, and the excess was being lost with out N salvage. In general, as N intake increases, there is a larger amount of urea-N being transferred to the rumen, but as a percentage of N intake more urea-N is transferred to the rumen on lower N diets.

Protein solubility: A paucity of work has been done to evaluate the impact of crude protein form (RDP vs. RUP) on N recycling. Ferrel et al. (2001) fed sheep a control diet (6.6% crude protein (CP) as a percentage of ration dry matter (DM)) and three isonitrogenous diets (11.2% CP as a percentage of ration DM) with urea, soybean meal, or a mixture of blood meal and feather meal as the N sources. They found that urea-N transfer to the GIT was 62.3, 47.2, 47.5, and 39.2% as a percent of hepatic release for the control, urea, soybean meal, and blood meal/feather meal diets, respectively. The concentration of N in the diet had the greatest effect on the amount of urea-N transfer to the GIT with the control diet having greater return than the three supplemented diets. However, the blood meal/feather meal diet, with higher RUP concentration compared with the urea and soybean meal with higher in RDP, resulted in the least urea-N transfer to the GIT. These findings are contradictory to the concept that high rumen ammonia concentrations inhibit urea entry to the rumen. However, Siddons et al. (1985) fed grass silage (32.1 g N/kg of DM and had 23.8 g of buffer soluble N per kg of DM) and dried grass (18.3 g N/kg of DM and 3.7 g of buffer soluble N per kg of DM) to sheep and found that sheep fed the silage had a net loss of N (4.0 g/d) between the mouth and the duodenum compared with sheep fed the dried grass who had a net gain (5.5 g/d) of N. The greater solubility of the N in the silage diets led to a higher concentration of rumen NH_3 possibly due to increased ruminal degradation. This higher ruminal ammonia

concentration could have limited urea-N entry into the rumen by inhibiting the urease activity of the rumen bacteria leading to a decrease in urea-N transfer to the rumen. Whereas the diet with the lower level of buffer soluble N had decreased ruminal ammonia and an increased potential for N return to the rumen. However, it is unclear as to whether these finding are a result of the differences in protein solubility or total protein concentration.

Carbohydrate digestibility: The digestibility of the carbohydrate in the diet affects route of urea-N entry into the rumen (through the rumen wall or saliva), and the relative proportion of urea-N transferred to the rumen or other sections of the GIT. Several studies have demonstrated that as the forage-to-concentrate ratio of a diet decreases there is greater urea-N transferred to the rumen as opposed to other sections of the GIT (Huntington, 1989; Huntington, 1996; Lobley et al., 2000; Reynolds and Huntington, 1988), with only one study (Lobley et al., 1996) observing no difference in urea-N entry into the rumen when diets differed in carbohydrate digestibility. Huntington (1989) found that when steers were fed a high concentrate diet, 45% of the urea that was synthesized by the liver went to the rumen, whereas only 7% of urea synthesized went to the rumen for steers fed an alfalfa diet. Lobley et al. (2000) compared a mixed concentrate-forage diet (30% barley, 50% hay, and 20% supplement) with an all-forage diet (50% grass hay pellets and 50% dried chopped hay), and observed a greater hepatic urea-N production and an increase in urea-N entering the GIT for sheep fed the concentrate-forage diet. Reynolds and Huntington (1988) reported that when lucerne was fed, the removal of PUN by the mesenteric-drained viscera (MDV) accounted for 83% of the portal-drained viscera (PDV) removal; whereas when a concentrate diet was fed, MDV accounted for only 32% of the PDV flux of PUN. This was reaffirmed by

Huntington et al. (1996), who observed low liver release of urea (94 mmol/h) when dietary concentrate was less than 20%, and peak release (146 mmol/h) was observed when dietary concentrate was 55%. The removal of the released N (PUN) by the MDV was negligible indicating that more urea was being removed by the rumen (Huntington et al., 1996). Also, steers that were fed steam-flaked versus dry-rolled sorghum had significantly increased urea flux to the PDV with a significant increase in recycling to the intestinal tissue (15 and 6 g/d, for steam-flaked and dry-rolled, respectively) and a numerical increase in recycling to the rumen (42 vs. 32 g/d for steam-flaked and dry-rolled, respectively; Theurer et al., 2002), with no difference in hepatic synthesis. However, again differences in forage to concentrate ratio are usually confounded by differences in protein content of the diet.

Urea Transporters: The control of expression of urea transporters in the rumen tissue is not well understood. It has been demonstrated that genetically they are more closely related to the rat kidney urea transporter (UT3) than other rat transporters or rabbit transporters (Rizthaupt et al., 1998). Recently Stewart et al. (2005) isolated and characterized urea transporter (UT-B) expression in bovine ruminal tissue (stratum granulosum, stratum spinosum, and stratum basale). The bovine urea transporter (UT-B2) is encoded for by a splice variant of the SLC14a1 gene. The gene encoding bUT-B2 is 20 Kb, has 10 exons, is found on chromosome 24, and shares 79% identity with the human UT-B transporter. Stewart et al. (2005) demonstrated that the transporter is bidirectional and can be inhibited by phloretin and mannitol. During inhibition, flux of urea across the rumen wall continued, albeit to a lesser extent, indicating that urea-N diffusion occurs. Marini and Van Amburgh (2003) hypothesized that when lower protein diets are fed urea transporter expression in the rumen increased to facilitate urea transfer

into the rumen. Consistent with this contention, they observed an increase in urea-N transfer to the rumen when low N diets were fed. However, expression of urea transporters (UT-A1, UT-A2, and UT-A4) was lower in the heifers fed the low-protein diets. Ritzhaupt et al. (1997) observed increased flux of urea from the mucosal to the serosal side of the rumen epithelial tissue with N depletion concomitant with no difference between transfer of urea from the serosal to the mucosal side. However, there was no replication in the Marini and Van Amburgh (2003) data set so the differences they observed can be attributed to animal variation. The Ritzhaupt et al. (1997) study was conducted in Ussing chambers, which may not be representative of in vivo conditions where the urea concentration in the blood in much higher than the concentration in the rumen forcing urea to flow from the blood to the rumen. Later work found no effect of diet on urea transporter expression in sheep fed diets ranging from 15.5 to 41.3 g of N/kg of dry matter (Marini et al., 2004).

Urea Utilization by Rumen Microbes

The transfer of more urea-N to the gut does not mean that more urea-N will be utilized by rumen microbes for microbial protein synthesis, as is desired. Marini and Van Amburgh (2003) found that as a proportion of urea-N that was recycled to the GIT, the percentage that returned to the ornithine cycle followed a quadratic relationship as N concentration of the diet increased with the peak at 40.7% for a diet containing 2.5 % N. The percentage that was excreted in the feces followed the opposite pattern, and there was no difference in the proportion that was utilized for anabolic purposes by the animal through breakdown of rumen microbial protein. Another study looked at the effects of a forage versus a mixed diet on N recycling and they also found that an increase in N transfer to the GIT did not necessarily equate to an increase in the amount of urea-N that

was utilized for anabolic purposes (Lobley et al., 2000). In order for N recycling to be of benefit to the cow, the urea that is returned to the rumen needs to be converted to microbial protein and then that protein needs to be broken down and absorbed further along the GIT. Although utilization for anabolic purposes implies use by the rumen microbes, it is possible that urea is broken down by rumen microbes attached to the rumen wall and the resulting ammonia is immediately absorbed and metabolized by the rumen epithelium (Abdoun et al., 2003), and this is not accounted for in the methods used in these studies.

Although there does appear to be some limitation to the use of recycled N, recycling still provides an important N source for rumen microbes, especially when fed low-N diets. Nolan and Leng (1972) reported that 80% of N incorporated into microbes comes from ruminal ammonia, and Archibeque et al. (2002) found that 59 to 66% of urea-N that enters the rumen is incorporated into microbial protein, which is available for anabolic use by the animal. Structural carbohydrate digesting bacteria utilize NH_3-N to a greater extent than bacteria that degrade nonstructural carbohydrates (Russell et al., 1992). Bryant and Robinson (1963) reported that cellulolytic bacteria preferred NH_3 to amino acids or peptide, however Atasoglue et al, (2001) recently showed that cellulolytic bacteria (*Fibrobacter succinogenes* BL2, *Ruminococcus albus* SY3, and *Ruminococcus flavefaciens* 17) prefer peptides and amino acids to NH_3 and if provided with these substrates proportionally more microbial N will be derived compared with NH_3. Starch degraders prefer amino acids and peptides, but may be able to utilize ammonia if that is the only available N source. Protozoa cannot utilize ammonia as a N source, but they add to the rumen ammonia N pool by producing ammonia as an intermediate in their N metabolism (Yokoyama and Johnson, 1988).

Rumen ammonia concentration determines which N assimilation pathway dominates. At lower ammonia concentration glutamine synthase pathway is the predominant pathway compared with the glutamate dehydrogenase pathway since it is a higher affinity system. The glutamate synthase pathway requires input of two ATP molecules to take two molecules of NH_4^+ to glutamate. The use of the more energy expensive pathway, along with the lack of N for microbial growth, at low ammonia concentrations may explain the minimum concentration of ammonia necessary to prevent inhibition of fiber digestion (50mg NH_3-N/L; Satter and Slyter, 1974).

Nitrogen Recycling and the Kidney

Initial blood filtration occurs as blood flows through the glomerulus. Urea is freely filtered through the glomerulus and the glomerular filtration rate, which is auto-regulated through vasoconstriction of the afferent and efferent arterioles, is the only control of urea filtration (Guyton and Hall, 2000). Low protein diets have been shown to reduce glomerular filtration rate by up to 21.6% after just one week leading to an increase in urea reabsorption by the kidney (Peil et al., 1990).

After the filtrate leaves the glomerulus it passes into the proximal tubule where 30 to 40% of filtered urea is reabsorbed (Guyton and Hall, 2000). From here filtrate enters the descending loop of Henle and there is additional urea reabsorption. Sodium reabsorption in the descending loop of Henle may impact urea reabsorption indirectly by increasing water reabsorption, which would lead to an increase in urea reabsorption due to the gradient formed. The filtrate then passes into the ascending loop of Henle and on to the collecting duct. The thick segment of the ascending loop of Henle and the distal tubule are impermeable to urea. Once the filtrate reaches the inner medullary collecting duct (IMCD) there can be significant urea reabsorption through urea transporters.

Urea is freely permeable across cell membranes, but due to the high filtrate flow rate through the collecting duct, diffusion alone is not rapid enough so transporters are necessary. Sands et al (1997) calculated that between 2 and 15 x 10^6 urea molecules move through an erythrocyte urea transporter per second and urea transport is slightly slower in the IMCD (0.3 to 1 x 10^5 per second; Kishore et al., 1997). On a relative basis this movement is very fast and is indicative of this transporter having a channel-like character. Also, active transporters may be needed to move urea against the concentration gradient to create the high concentration of urea in the medullary interstatium, which is needed to concentrate urine (Sands et al., 1997). The urea concentration in the medullary interstatium is greater than the urea concentration in the loop of Henle due to urea reabsorption by urea transporters. This allows for greater water and sodium absorption from the ascending loop of Henle leading to more concentrated urine.

Sands et al. (1987) found that the IMCD can be separated into two distinct functional sections. The initial IMCD has low permeability to urea and is not stimulated by vasopressin whereas the terminal IMCD is very permeable to urea and is responsive to vasopressin (Sands et al., 1987). This functional difference in permeability probably helps to maintain the urea gradient in the medullary interstatium allowing for urine concentration. Addition of vasopressin to isolated IMCD cells increases urea permeability rapidly at first followed by a slower increase (Nielson and Knepper, 1993) and this process appears to work through the second messenger cAMP (Star et al, 1988). Vasopressin increases the production of cAMP, which increases the production of protein kinase A resulting in increased phosphorylation of UT-A1 and increased urea transfer into the medullary interstatium (Zhang et al., 2002). It is unclear whether the

phosphorylation of UT-A1 causes an increase in the quantity of transporters in the membrane or the activity of the transporter. Nielson and Knepper (1993) speculated that urea transporters are constantly being inserted and removed from the cell membranes and that the initial rapid increase was due to a increase in insertion followed by a decrease in removal of the transporters (Gillian and Sands, 1992). This would increase the V_{max} but not the K_m, which is reflective of a system where increasing the urea concentration would not lead to saturation (Chou et al., 1990). Isozaki et al. (1993) also reported increased complexity of the basolateral membrane when low protein diets were fed indicating insertion of transporters had occured.

Creating a hyperosmolarity situation, as is present in the IMCD region, also increases the permeability of IMCD to urea by increasing the intracellular calcium concentration (Gillian et al., 1993). Why an increase in intracellular calcium increases urea permeability is unclear. However, regardless of how it works increasing vasopressin levels in a hyperosmolar environment has an additive affect on increasing urea reabsorption (Gillian et al., 1993).

It is well known, that urea is critical to the ability of the kidney to concentrate urine. Dogs fed a low protein diet were not able to concentrate urine to the same extent as dogs fed a high protein diet due to the decrease in urea concentration in the interstitial medullary space (Levinsky et al., 1959). However, this decrease in urea concentration in the interstitial medullary space is not due to a decrease in urea reabsorption; in practice the opposite case is true with urea reabsorption from the IMCD increasing with lower protein diets (Isozaki et al., 1994). Rats fed a low protein diet were able to increase urea reabsorption with the addition of vasopression and cAMP so the low protein diets did not impact vasopressin's ability to stimulate cAMP production. Therefore, the low protein

concentration of the diet impacts either new transporter synthesis or insertion into the membrane (Isozaki et al., 1994). A similar response has been observed in sheep where a 1.45% N diet was fed and 47% of filtered urea was reabsorbed whereas in sheep fed a 3.40% N diet only reabsorbed 8% of the filtered urea (Marini and Van Amburgh, 2003). However, this increase in urea reabsorption from the kidney was not accompanied by an increase in urea transporter abundance in the kidney (Marini et al., 2004). This is in contradiction to the findings of Nielson and Knepper (1993) who found that an increase in urea reabsorption at the IMCD was due to an increase in urea transporters. It is unclear as to why Marini et al. (2004) were able to see an increase in urea reabsorption with no difference in urea transporter abundance, but one explanation could be their method of isolating the kidney material. The more inner segment of the medulla would have a higher urea concentration, and the adjacent IMCD would have more active urea transporters compared with the more outer segments of the medulla (Sands et al., 1987). Therefore, a tissue sample taken from the outer segment of the medulla would not contain the most active urea transporters. The increase in urea re-absorption by the IMCD in animals fed a low protein diet is somewhat conflicting with previous data (Levinsky et al., 1959) that showed a decrease in the urea concentration in the medulla. One explanation for the increase in re-absorption without an increase in concentration in the medulla is that the blood (vasa recta) is picking up that urea and taking it to other parts of the body (rumen) instead of letting it be re-secreted into the ascending loop of Henle.

Double-labeled [^{15}N]Urea Method

The double-labeled [^{15}N]urea method, developed in humans (Jackson et al., 1993), is more accurate and less invasive than methods that involve single labels and multiple catheters. In this method doubly-labeled urea ([^{15}N^{15}N]urea) is infused into the blood and

the ratio of singly labeled urea ([^{15}N^{14}N]urea) to doubly-labeled urea is a measure of N recycling. There are two main routes that singly-labeled urea can be formed. One is from the breakdown of the doubly-labeled urea in the rumen to ammonia, which is then absorbed into the blood and converted to urea. The other is by digestion and absorption of microbial protein, which had incorporated the labeled N. Also, by measuring the amount of labeled N in the feces the fraction of urea-N that was transferred to the GIT and subsequently utilized for anabolism, returned to the ornithine cycle by re-absorption, or excreted can be determined. Sarraseca et al. (1998) were the first to use this model in sheep and it was later corrected by Lobley et al. (2000) to adjust for multiple recycling of the same N to the rumen. Previous models developed in humans assumed only one N cycle (Jackson et al., 1993) where urea produced by the liver was broken down in the large intestines to ammonia. The ammonia-N was then either excreted directly or absorbed and incorporated into another urea molecule, which was then excreted in urine. The Lobley et al. (2000) correction allows for this newly formed urea to be transferred to the GIT instead of assuming that it was excreted.

Although this method is a major step forward in urea-N recycling research, one problem that it has it that it does not account for ammonia metabolism by the rumen epithelium, which has been observed in sheep (Abdoun et al., 2003). The model assumes that the rumen microbes must have utilized any N that was not returned to the blood for anabolic purposes, which leads to an over-prediction of urea-N used for anabolism. Also, previous work studying N recycling, including the newer double-labeled [^{15}N]urea method, was all done in sheep, non-lactating heifers, or beef steer, therefore milk urea-N loss was not considered.

Incorporation of Urea-N Recycling Into Ration Formulation Models

In many studies, urea-N synthesis by the liver is equal to, or greater than, the amount of N apparently digested (Lobley et al., 2000, Sarreseca et al., 1998, Reynolds et al., 1991). This means that unless urea-N is transferred to the rumen for microbial protein synthesis these animals would be in a negative N balance. The Cornell model (Russell et al., 1992) takes N recycling into account by factoring it into RAN, using CP intake to predict N recycled ($Y = 121.7 – 12.01X + 0.3235X^2$, where Y = urea-N recycled as a percentage of N intake and X = intake of CP as a percentage of diet DM; NRC, 1985). However, CP intake is not the only factor that influences recycling of N in ruminants. Also, this model does not estimate how much urea-N is available to the rumen microbes, or how that urea-N is utilized. The NRC (1989) predicts urea-N recycling as 15% of the intake protein requirement (rumen influx protein = [(rumen available protein + undegraded intake protein)/(1.15)] x 0.15). The NRC (2001) evaluates urea-N recycling differently and assumes that there is no net urea-N recycling (N influx = N efflux). This assumption is used in the RDP requirement prediction equations, which are then used to predict the RUP requirement. However, under certain feeding conditions the net urea-N recycling is not likely to be zero. For example, when low protein diets are fed there is increased salvaging of urea by the kidney and increased reabsorption of N into the GIT from the blood resulting in greater N flow from the rumen than what was fed. In this case the current NRC (2001) would be over-predicting the RDP requirement because it is not accounting for the N supplied by recycling. With the increased emphasis placed on feeding lower protein diets to cows to reduce N excretion it is possible that the RDP requirement is being over-predicted in some situations.

Conclusions

Although urea-N recycling is fairly well understood in other animals the flow rates of urea-N within lactating dairy cows, and how those flows may be influenced by diet have not yet been determined. Due to this lack of data, the NRC (2001) does not include urea-N recycling in its RDP prediction equations even though use of recycled urea-N for microbial protein production could impact the RDP requirement. Uncertainties in the RDP prediction values leads to overfeeding of N, which can lead to environmental pollution and increased feed costs. Therefore, the objectives of this research were to determine the effect of RDP and RUP on urea-N recycling, determine if urea-N recycling is being regulated in lactating dairy cows, and if so where is that regulation occurring (kidney or rumen), determine how much microbial protein is derived from recycled urea-N, and lastly evaluate the NRC 1989 and 2001 predictions of N flows from the rumen and ruminal N balance.

CHAPTER 2

EFFECT OF DIETARY RUMEN UNDEGRADABLE AND RUMEN DEGRADABLE PROTEIN CONCENTRATION ON NITROGEN BALANCE AND MICROBIAL NITROGEN FLOW IN MID-LACTATION COWS

ABSTRACT

The effect of rumen degradable protein (RDP) and rumen undegradable protein (RUP) on nitrogen balance and rumen outflow of microbial N in lactating dairy cattle was investigated. Eight mid-lactation Holstein cows were assigned to a repeated 4 x 4 Latin square, balanced for carryover effects. The diets were isoenergetic and contained 16.7, 18.2, 19.8, and 20.6% crude protein (CP) as a percentage of dry matter (DM). Diets were arranged in a 2 x2 Factorial with two levels of RDP (10.0 and 12.5% of ration DM) and two levels of RUP ((5.6 and 8.0% of ration DM). Cows fed the 12.5% RDP diet tended to produce less milk ($P = 0.06$) with increased milk protein concentration ($P < 0.05$). Cows fed the 12.5% RDP diets had increased ruminal propionate concentrations and increased DM and neutral detergent fiber digestion. The 8.1% RUP diets resulted in increased fecal N excretion presumably due to greater flow of bypass protein to the feces. Urinary N excretion increased with increasing RDP and RUP yet with an increase in urine volume. Similar urinary N concentrations were observed across diets. High RUP diets tended to increase liquid flow out of the rumen ($P = 0.06$), N flowing with the liquid fraction ($P = 0.09$), and liquid associated bacterial N ($P = 0.10$). Potential transfer of excess RUP to the rumen in the form of urea-N did not increase microbial N flow out of the rumen. Rumen degradable protein did not affect flow of total N or microbial N out of the rumen. Altering the RDP and RUP concentration of diets when the total crude protein concentration is above the requirement had little effect on production parameters. However, N balance and microbial flow out of the rumen were affected. The National Research Council (NRC) 1989 model predicted negative rumen N balance across all four diets with no mean or linear bias, while the NRC 2001 predicted positive rumen N balance for all diets with a significant mean bias. Ultimately though, the NRC 2001

model predicted a higher RDP requirement and a lower RUP requirement compared with the NRC 1989. Both models reflect the dietary changes that were intended by increasing the RDP and RUP in a factorial manner. The NRC 1989 predicted flow of microbial N and total N from the rumen more accurately than the NRC 2001.

INTRODUCTION

Understanding microbial and whole animal metabolic N utilization can aid in formulating dairy rations for optimum efficiency. Such a ration would provide N in a way that meets animal requirements and reduces N loss, ultimately mitigating both economical and ecological problems associated with N lost to the environment via feces and urine. Rumen degradable protein (RDP) and rumen undegradable protein (RUP) are central sources of N in dairy rations, and their interaction impacts the protein ultimately available to the cow from the rumen. This paper will focus on the effect of RDP and RUP on whole animal N balance and microbial N flow out of the rumen in lactating dairy cows.

Rumen degradable protein and RUP have differing fates in the rumen. Rumen degradable protein supports microbial protein synthesis by direct degradation to amino acids and indirectly by recycling of RDP-N to the rumen as urea-N. Rumen undegradable protein bypasses rumen digestion and following digestion and hydrolysis by mammalian enzymes supplies amino acids directly to the cow. Some amino acid-N from RUP will be incorporated into urea through the ornithine-urea cycle. Therefore, RUP-N can ultimately supply N for microbial protein synthesis through the ornithine-urea cycle, and the urea-N formed is subsequently transferred to the rumen. Lobley et al. (1995) theorized that amino acid-N incorporation into urea-N would negatively impact the N economy of the animal because one of the N in urea is supplied by aspartate. A

study by Moorby and Theobald (1999) refulted Lobley et al. (1995) by demonstrating no negative effect of urea synthesis on amino acid availability for milk production. A possible explanation for this is RUP-N may alleviate a RDP deficiency and support microbial protein synthesis via urea-N without negatively impacting the N economy of the cow.

Microbial protein accounts for 65% of non-ammonia N flow from the rumen (Reynal and Broderick, 2005). Microbial protein is derived from ammonia-N (Nolan and Leng, 1972), but microbial protein synthesis increases when amino acids are available in addition to ammonia-N (Hungate, 1966). Fiber-degrading bacteria readily utilize ammonia-N as a N source, where as concentrate degraders prefer amino acids and peptides for microbial protein synthesis (Russell et al., 1992). Thus, dietary factors that inhibit fiber-degrading bacteria in the rumen will decrease the efficiency of urea-N use and unused urea-N will be excreted in the urine.

The ability of the rumen microbes to utilize both feed-N and recycled urea-N for microbial protein production increases overall N utilization by the ruminant. As these microbes ultimately supply protein to the cow to support milk production the nutrient requirement prediction models (NRC, 1989 and NRC, 2001) must accurately predict the dietary RDP required to optimize microbial protein synthesis, given that RDP is not the only N source utilized for microbial protein synthesis. Ultimately, prediction of microbial protein flow from the rumen cannot be accurate without better understanding of these interactions. The NRC 1989 directly accounts for recycled urea-N in its microbial N flow and RDP requirement prediction equations. In contrast, the NRC 2001 assumes no net recycling to the rumen, and does not account for it in predicting microbial N flow or the RDP requirement. Both the NRC 1989 and NRC 2001 use microbial protein flow to

predict the RDP requirement. Therefore, accurately predicting microbial N flow from the rumen is also necessary to avoid overfeeding RDP in order to minimize N excretion (St-Pierre and Thraen, 1999).

The objectives of this study were to determine the effect of RDP and RUP on microbial N flow and N balance, and to evaluate the NRC 1989 and NRC 2001 predictions of N flow from the rumen and rumen N balance. Our hypothesis is that diets low in RDP will lead to decreased microbial N flow and N balance when the diet also contains low RUP, but increasing the RUP content of the diet will result in increased transfer of N (through recycled urea-N) to the rumen and enhanced microbial N flow and N balance. Further, use of RUP may be improved if fed concomitantly with a low RDP content diet improving N utilization overall. When RDP is insufficient, diets with more RUP may recycle more N to the rumen, and result in greater microbial growth and enhanced digestion.

MATERIALS AND METHODS

Cows and Diets

Eight ruminally fistulated Holstein cows from the USDA-ARS in Beltsville, MD were divided into two groups based on DIM. The first group average 156 DIM and the second group averaged 152 DIM at the start of the trial. The experiment was designed as a repeated 4 x 4 Latin square with 21-d periods with sampling occurring during the last 2 days of each period. Cows were housed in a tie stall barn with ad libitum access to water. The diets were fed twice daily at 1400 h and 2000 h for 10% refusal. The cows were milked twice daily at 800 and 2000 h. The experiment was conducted with the approval of the University of Maryland Animal Care and Use Committee and the Beltsville area Animal Care and Use Committee, USDA.

Diets (Table 2-1) were arranged in a 2 x 2 Factorial with two levels of RDP (10.0 and 12.5 % of ration DM) and two levels of RUP (5.6 and 8.0 % of ration DM). This led to the following treatment diets: low RDP/low RUP, low RDP/high RUP, high RDP/low RUP, and high RDP/high RUP. All the diets contained similar amounts of corn silage, grass hay, ground corn, and Megalac® (Church and Dwight Co., Princeton, NJ). Soybean meal and urea were the main RDP sources, and Soypass® (Ligno-Tech USA, Inc., Rothschild, WI) was the main RUP source in the high RUP diets replacing soybean meal and cornstarch.

Markers

The three markers used in this experiment were Cr-mordanted neutral detergent fiber (NDF), Co-EDTA, and [^{15}N^{15}N]urea (98 atom % ^{15}N^{15}N, Cambridge Isotope Laboratories, Inc., MA). The Cr-mordanted NDF and the LiCo-EDTA were produced according to the procedure of Uden, et al. (1980). The Cr-NDF (3.69 g of Cr-mordanted NDF equal to 0.24 g of Cr) and the Co-EDTA (154 mg of Co-EDTA dissolved in 10 mL of distilled water) were dosed directly into the rumen every 4 hours starting at 1200 h on day 15 and continuing to the end of each period. The Cr-mordanted NDF and Co-EDTA served as solid and liquid passage markers, respectively. On day 19, the cows were fitted with 16 G x 36 inch intravenous catheters (Delmed, Inc., New Brunswick, NJ) for infusion of the [^{15}N^{15}N]urea. The [^{15}N^{15}N]urea was dissolved in 9% sterile saline solution (4.17g of urea/L) filtered through 0.22 μm filter (Millipore, Bedford, MA), and infused directly into the jugular vein of the cow at a rate of 20 mL/h beginning at 1200 h on day 19 and continuing through the end of the period to supply 2.1 g/d of [^{15}N^{15}N]urea. It was determined from our modeling and previously reported data (Lobley et al., 2000, Reynolds et al., 1988) that 48 h infusion of [^{15}N^{15}N]urea is a significant time to reach a

constant enrichment in the urinary doubly-label urea pool, but not the [^{15}N^{14}N]urea pool. The ^{15}N concentration in microbial N (not in steady state), which derived from the recycled [^{15}N^{15}N]urea, was used to measure microbial N flow. The concentration of [^{15}N^{15}N]urea and [^{15}N^{14}N]urea in the urine was used to calculate urea recycling (reported in Dinh et al. submitted)

Total mixed ration (TMR), orts, and diet ingredient sampling

Corn silage, grass hay, TMR, and grain mixes were sampled weekly, composited over each period, dried at 65°C, and ground through a Wiley Mill (1-mm screen; Arthur H. Thomas Co., Philadelphia, PA). Orts were collected daily and 3% was kept to create a weekly composite, which was composited over each period, dried, and ground. Corn silage, grass hay, TMR, orts, and grain mixes were analyzed for total N by Dumas combustion (Leco FP-428, Leco Corp., St. Joseph, MI). Amylase-modified NDF and ADF were conducted according to Van Soest et al. (1991) with modifications by Mertens et al. (2002), and acid detergent lignin (Goering and Van Soest, 1970) were analyzed sequentially, and neutral detergent insoluble N, acid detergent insoluble N, starch (Karkalas, 1985), DM (100°C overnight), and ash (600°C for 4 hours) were also analyzed.

Rumen sampling

Rumen samples were taken prior to the start of the [^{15}N^{15}N]urea infusion and every 4 hours for the last 24-h or each period starting at 1200 h on day 20. Approximately 250 mL of rumen fluid was collected using a 1.5 cm diameter PVC pipe attached to a vacuum pump and filtered through 1 mm mesh as the sample was being taken. The pH of the sample was immediately recorded and the sample was aliquoted into 3 50-mL tubes with one tube containing 1 mL of 50% HCl. The tubes were immediately frozen at –20°C for further analysis of rumen ammonia N (**RAN**) and VFA.

Rumen ammonia N was determined according to Broderick and Kang (1980) on a 20 fold dilution of rumen fluid (diluted in 1 mL of HCl) in a 96-well plate with 25 μL of sample, 100 μL of phenol-nitroprusside-tartrate, and 100 μL of alkaline hypochlorite were added to each well. The plate was shaken, allowed to sit for 30 minutes, and read on Sunrise spectrophotometer (Phenix Research Products, Hayward, CA) at 570 nm. The concentration of ammonia was calculated from regression of a standard curve.

Reticulum sampling

Reticulum samples were acquired at the same time as the rumen samples using the same PVC and vacuum device except the samples were not filtered through the 1 mm mesh. Approximately 250 mL of fluid and particles were removed from the reticulum and immediately weighed. The sample was poured through a set of sieves (2mm, 1mm, 0.5 mm, and 0.25 mm) into a collecting pan. The fluid in the collecting pan was weighed and transferred to a separate container. The sieves were rinsed with water and the material remaining on each sieve was weighed, frozen, and later dried at 65°C. A portion of the dried sample was used to create a composite over each period for each sieve size. The fluid from the collecting pan was centrifuged at 500 x g for 15 min at 4°C forming a pellet of solid associated bacteria (**SAB**) and feed. This pellet was weighed, immediately frozen, and later freeze-dried and a proportion was composited over each period. The supernatant was re-centrifuged at 4640 x g for 30 min at 4°C forming a pellet of liquid associated bacteria (**LAB**). A sample of the supernatant from this centrifugation was immediately frozen, weighed, and later freeze-dried, with a portion composited over each period. The pellet of LAB was weighed, immediately frozen, later freeze-dried, and analyzed for N and ^{15}N (University of California – Davis Stable Isotope Facility, Davis, CA). The period composites of the sieve samples (2 mm, 1mm, 0.5 mm, and 0.25 mm),

the SAB/feed pellet, and supernatant from the spin that resulted in the LAB pellet were analyzed for Cr and Co concentrations according to the nitric-perchloric digestion method of Perkin-Elmer (1982), and the concentration of Cr and Co was determined by atomic absorption spectrophotometry (Perkin-Elmer 5100PC Atomic Absorption Spectrophotometer). Samples were then pooled within sampling time across sieve size and SAB/feed pellet according to their flow rates and analyzed for ^{15}N and total N (University of California – Davis Stable Isotope Facility, Davis, CA). Because the ^{15}N concentration was not at steady state in the rumen microbes it was necessary to measure it on composite samples from each time point. The ^{15}N concentration for the LAB and the SAB/feed and sieve composites were adjusted for natural background concentrations according to the following equation:

$$^{15}N\text{-APE} = {}^{15}N \text{ atom } \% - {}^{15}N \text{ atom } \% \text{ in the natural background}$$

The ^{15}N natural background was measured on samples that were taken prior to the start of [$^{15}N^{15}N$]urea infusion for each period.

Urine and fecal sampling

Indwelling Foley catheters with a 75-cc balloon (C. R. Bard, Inc., Covington, GA) were used for 24-h total urine collection. The catheters were placed in the cows at 1200 h on day 20 and were removed at 1200 h on day 21. Urine was collected into 25-L plastic carboys containing 875 mL of 50% HCl to keep the pH below 3. After the catheters were removed the urine was mixed thoroughly and a 1-L sample was collected and frozen for further analysis. Collection pans were placed behind each cow at 1200 h on day 20 and removed on day 21 to collect all feces over a 24-h period. The feces were weighed, mixed, and sub-sampled immediately. The fecal samples were dried at 65°C and subjected to the same analyses as the feed samples.

Milk samples

Milk yield was recorded for both the AM and PM milking over the entire course of the experiment, but only the weekly average for the last week of each period was used for data analysis. Milk samples were taken during the evening milking on day 19, both milkings on day 20, and the morning milking on day 21. A composite sample for each period was sent to Lancaster DHIA (Manheim, PA) for determination of protein, fat, solids non-fat, MUN, and somatic cell count.

Volatile fatty acid analysis

Rumen fluid samples were prepared for VFA analysis by centrifuging 1 mL of rumen fluid at 7,000 x g for 10 minutes. Sub-samples of the supernatant (0.4 mL) were placed in separate 1.5-mL tubes and 0.1 mL of internal standard (2.4 g/L of 2-ethylbutyric acid in 25% o-phosphoric acid) was added to each. The samples were vortexed for 30 seconds and 0.1 mL of each was transferred to 1.5-mL GC vials with glass inserts. Concentrations of VFA were determined by gas chromatography with reference to a standard curve using the Agilent 6890 series GC and 7683 series injector (Agilent Technologies, Inc., Santa Clara, CA) with a 4 mm column that was 1.8 m in length and packed with 100/120 Chromosorb® (Supelco, Inc., Bellefonte, PA). Helium was used as the carrier gas at a rate of 25 mL/min. The front inlet temperature was 180°C, the oven temperature was 150°C for 12 minutes, and the flame ionization detector temperature was 180°C.

Calculations

Dry matter and liquid flow rate: Dry matter and liquid flow out of the rumen were calculated from the concentrations of Cr in the SAB/feed pellets and each sieve sample composited over each period, and liquid flow rate from the Co in the supernatant

samples composited over each period. It is assumed that neither the Cr-mordanted NDF nor the Co-EDTA were absorbed or degraded in the rumen, therefore, what was dosed into the rumen flowed out of the rumen. Based on previous experiments in our lab (Peterson, 2006) a dosing schedule for the Cr-mordanted NDF and the Co-EDTA of every 4 hours for 4 days prior to sampling was determined to be adequate to reach steady state conditions in the rumen. Therefore, the differential equation of

d[Cr]/dt = g/d of Cr dosed – k_s*([Cr] in the rumen) can be simplified to

g/d of Cr dosed = k_s*([Cr] in the rumen) and rearranged to

fractional flow rate for solids (k_s) = g/d of Cr dosed/([Cr] in the sieve and SAB/feed pellet sample divided by % each sieve and SAB/feed was of the total DM).

The same equation holds for the determining the flow rate of the liquid fraction out of the rumen by substituting the Co concentration in the supernatant for the Cr concentration in the sieves and SAB and using the Co dosing rate in place of the Cr dosing rate. The flow of N associated with the liquid and solid fraction was calculated by multiplying the liquid and solid flow rates by the amount of N in those respective fractions.

*Microbial N (**MN**) flow rate:* The flow of microbial N out or the rumen was determined from the liquid and solid flow rates, the ^{15}N-atom percent excess (**APE**) concentration in the LAB pellet from each sampling time and the ^{15}N-APE in the composite samples (the four sieve fractions and the SAB/feed within each sampling time; composited according to flow data) of each time point according to the following equation:

[(^{15}N-APE from the sieve and SAB/feed composite sample) x N concentration in the sample]/sample wt (g) = g ^{15}N/g DM

[g ^{15}N/g DM x solid flow rate]/^{15}N-APE from the fast pellet sample = flow of microbial N leaving the rumen (g/d) at that time point, which is associated with the solid fraction (all the sieve fractions plus the material from the SAB/feed pellet).

The flow of the liquid associated bacteria was determined in a similar fashion except the ^{15}N-APE from the fast pellet was used in place of the ^{15}N-APE in the sieve fractions and SAB/feed pellet and the liquid flow rate was used in place of the DM flow rate. The mean microbial N flow rate for each period was calculated by averaging the microbial N flow rates at each sampling time within each period.

Apparent ruminal and total tract digestibility: Apparent total tract digestibility of DM was calculated using DMI from day 20 of each period:

Apparent total tract digestibility = [1- (fecal dry wt (kg/d)/DMI (kg/d))]

Apparent total tract digestibility of NDF and N were calculated similarly according to the following equation

Apparent total tract digestibility = [1 – (fecal NDF (kg/d)/ NDF intake (kg/d))]

Apparent ruminal digestibility of DM and degradability of N was calculated according to the following equation:

Apparent ruminal DM digestibility = [1 – (DM flow from the rumen (kg/d)/DMI (kg/d)]*100

Apparent ruminal N degradability was calculated in the same manner except feed N flow (total N flow – MN flow) and N intake were used.

Statistical analysis

Data were analyzed using the Mixed procedure of SAS (2002) according to the following models:

$$Y_{ijklm} = \mu + G_i + C(G_j) + P(G_k) + RDP_l + RUP_m + (RDP * RUP)_{lm} + e_{ijklm}$$

Where,

μ = overall mean, G_i = effect of group (i = 1, 2), $C(G_j)$ = random effect of cow within group (j = 1, 2, 3, 4), $P(G_k)$ = effect of period within group (k = 1, 2, 3, 4), RDP_l = effect of level of RDP (l = 1, 2), RUP_m = effect of level of RUP (m = 1, 2), $(RDP * RUP)_{lm}$ = effect of interaction of RDP and RUP, and e_{ijklm} = residual error, assumed to be normally distributed.

The effect of dietary CP concentration was evaluated according to the following model:

$$Y_{ijkl} = \mu + G_i + C(G_j) + P(G_k) + T_l + e_{ijkl}$$

Where,

μ = overall mean, G_i = effect of group (i = 1, 2), $C(G_j)$ = random effect of cow within group (j = 1, 2, 3, 4), $P(G_k)$ = effect of period within group (k = 1, 2, 3, 4), T_l = dietary crude protein effect (l = 1, 2, 3, 4), and e_{ijkl} = residual error, assumed to be normally distributed.

Evaluation of NRC 1989 and 2001 models

All predictions for both NRC 1989 and 2001 models were based on observed dry matter intake and milk production. The absorbed protein (**AP**) value was calculated according to the NRC (1989) except that microbial CP was estimated as a fraction of RDP when diets were deficient in RDP based on the NRC 1989 text, where microbial CP (g/d) = 0.9 x (RDP + 0.15 CP intake). For the NRC 2001, the RUP requirement was calculated based on the assumption of adequate RDP. All other RDP, RUP, and MP requirement values were calculated according to the previously described models (NRC, 1989; NRC, 2001). The MN and the RUP-N flowing to the duodenum were calculated according to the NRC (1989) and NRC (2001), and the total N flow to the duodenum was the sum of MN and RUP-N.

The models were evaluated against observed rumen N flow and N balance data. The residuals, mean bias, linear bias, and root mean square prediction error (**RMSPE**) were calculated according to the following equations.

Residuals = observed – predicted

$RMSPE = sqrt[(\Sigma(residuals^2))/n]$

$Mean\ bias = [\Sigma(residuals)]/n$

$Linear\ bias = r^2 \times (RMSPE^2 - mean\ bias^2)$

Where r^2 was determined by plotting the residuals versus the predicted. Mean bias was deemed significant if it was different from zero at $P < 0.05$, and the linear bias was deemed significant if the slope of the line of the residuals versus predicted plot was different from 0 at $P < 0.05$.

The residuals and the model predictions were analyzed using the Mixed procedure of SAS (2002) according to the previously described model. Significance was declared at $P < 0.05$ for all data analyzed.

RESULTS AND DISCUSSION

Dietary Composition

Dietary composition is listed in Table 2-1. The diets ranged in CP concentration from 16.7 to 20.6% as a percentage of ration DM, which were higher than expected due to greater than anticipated CP concentration of the grass hay. The RDP and RUP concentrations were estimated from the NRC (2001). The RDP concentration as a percentage of ration DM was 10.0% for the low RDP diets and 12.5% for the high RDP diets. The RUP concentration for the low RUP diets was 5.6% and 8.0% for the high

RUP diets as a percentage of ration DM. The NDF, ADF, and lignin composition was similar between diets at 32%, 15%, and 2% as a percentage of ration DM, respectively.

Milk Yield and Milk Composition

Cows fed the high RDP diets tended to have decreased milk yield ($P = 0.06$) and decreased 4% FCM ($P = 0.09$: Table 2-2) compared with cows fed the low RDP diets. Cows fed the high RUP diets were less efficient at converting DM intake to milk yield (kg/kg: $P = 0.01$). Cows fed the high RDP diets had significantly higher milk true protein concentration, but there was no effect of RDP or RUP on milk protein yield due to the small difference in protein concentration coupled with opposite numerical differences in milk yield. Dunlap et al. (2000) reported no effect of RUP on milk and protein yield and concentration, or milk fat yield or concentration even in diets that were below the NRC (2001) RUP requirements. All of our treatment diets were above the NRC (2001) RUP requirement, therefore, we would expect no effect of RUP on milk yield or protein concentration. Reynal and Broderick (2005) also reported no effect of RDP on milk yield with a similar RDP range (10.6 to 13.2% of ration DM). There was no effect of RUP or RDP on milk fat or solids non-fat concentrations or yields.

Rumen Metabolism

The RAN concentration was elevated ($P < 0.01$) for cows fed the high RDP diets (Table 2-3). Cows fed the low RDP diets exhibited RAN concentrations close to those expected to be limiting for microbial protein production (Satter and Slyter, 1974), which could impact ruminal digestion and use of recycled urea-N. There was a significant interaction of RUP and RDP for acetate:propionate ratio being higher on the high RUP diet when cows were also fed in combination with low RDP, but not when high RDP was fed. Ruminal propionate concentration was greater for cows fed the high RDP diets, but

neither RDP nor RUP affected the other individual VFA concentrations, total VFA concentration, or ruminal pH.

Intake and Digestibility

Dry matter intake (Table 2-4) was highest for cows fed the high RUP diets, which coincided with the numerically greater milk yields. Neutral detergent fiber intake was greater for cows fed the high RUP diets, also reflective of the increased DMI for these diets as NDF concentration was not different across the four diets.

Total tract dry matter digestibility (Table 2-4) was high for cows fed all four diets with cows fed the high RDP diets having the greatest total tract apparent digestibility (77.0%, $P = 0.03$), but there was no difference in ruminal DM digestibility (due to insufficient amount of sample we were unable to determine ruminal NDF digestibility). All four diets contained low concentrations of ADF ranging from 14.4 to 15.9% as a percentage of ration dry matter. Lignin concentration of these diets was also low ranging from 0.8 to 1.9% as a percentage of ration dry matter. Lignin and ADF are inversely related to dry matter digestibility (Danley and Vetter, 1973), which explains the high total tract digestibility of our diets. Although our DM and NDF digestibilities are higher than what has been reported by others (Ivan et al., 2005, Shaver et al., 1988, and Weiss and Wyatt, 2002), Robinson and McQueen (1992) fed diets where good timothy hay silage replaced poor timothy hay silage and the apparent whole tract DM digestibility ranged from 75.8 to 78.2% and the NDF digestibility ranged from 64.2 to 69.7%.

The NDF digestibility was higher for cows fed the high RDP diets possibly due to the higher RAN concentration supporting a larger fiber degrading bacterial population. Fiber degrading bacteria have a preference for ammonia-N over preformed amino acids and peptides (Russel et al., 1992), and it is possible that the low RAN concentrations

observed for the low RDP diets was enough to inhibit the fiber degrading bacteria leading to decreased ruminal NDF digestion. Total tract CP digestibility was also highest in cows fed the high RDP diets further indicating that microbial fermentation was inhibited in the low RDP diets. We expected that the low RDP/high RUP diet would result in higher CP digestibility compared with the low RDP/low RUP diet, but the opposite was observed. This could be explained by higher DMI for cows fed these diets leading to faster passage rates through the gastrointestinal tract and decreased digestion (Allen and Mertens, 1988).

Nitrogen Excretion and Balance

Urinary N excretion and N intake increased with increasing dietary N (Table 2-5). All four diets had higher than the NRC (2001) recommended dietary CP concentration of 16.5% resulting in increased N excretion of the excess N. Unlike urine, the majority of milk N is found in the protein fraction, not the urea fraction, and the slight increase in protein concentration in cows fed the high RDP diets was not enough to lead to an effect of RDP and RUP. Fecal N excretion was higher for cows fed the high RUP diets. Conrad et al. (1960) reported that microbial protein only accounts for 14% of fecal protein, therefore, the differences we observed in fecal N excretion were likely due to the increase in feed protein escaping ruminal and intestinal digestion rather than differences due to increased microbial growth from recycling of urea-N to the lower digestive tract.

There was no effect of RDP or RUP on N balance. However, the dietary CP range evaluated in this study (3.9 percentage units) was much smaller than the range evaluated by Marini and Van Amburgh (2003; 12.2 percentage units) where dietary CP did affect N balance. Therefore, the small dietary CP range across our diets prevented us from observing an effect of dietary CP on N balance. We were able to observe that cows

fed the low RUP diets were more efficient at utilizing intake N for milk N. Olmos Colmenero and Broderick (2006b) also observed greater N utilization efficiency when cows were fed diets with lower N concentrations.

Nitrogen and Microbial N Flow From the Rumen

There was a tendency for cows fed the high RUP diets to have increased liquid flow rate out of the rumen ($P = 0.06$), which represents the flow rate of the supernatant fraction that was produced after the high-speed centrifugation (Table 2-6). The greater liquid flow rate resulted in greater LAB-N flow rate as well ($P = 0.10$). Urine weight was also increased for cows fed the high RUP diets indicating greater water consumption, which could explain the increased liquid flow rate. There was no effect of RDP or RUP on the flow rate of DM (composite of sieve fractions and SAB/feed pellet), total flow (liquid plus solid flow rate), RUP-N flow, total N flow (RUP-N plus MN), SAB-N flow, or MN flow out of the rumen. Moscardini et al. (1998) observed no effect of increasing RUP on microbial N flow as measured by urinary purine derivatives, and Olmos Colmenero and Broderick (2006a) reported no effect of dietary CP concentration on omasal flow rate of liquid or solid associated bacteria and total bacterial N in cows fed diets with CP concentration comparable to our study. Averaged across the four treatments, 63% of microbial N was flowing with the solid fraction with microbial N accounting for 64% of the total N flowing from the rumen, which agrees with the 57% of microbial N flowing with the solid fraction and microbial N accounting for 65% of the total NAN flowing, reported by Reynal et al. (2005). A negative ruminal N balance (N intake (g/d) – N outflow (g/d)) was observed for all four diets, where N outflow represented feed N and MN outflow. Urea-N transferred to the rumen and utilized by the

rumen microbes would flow out of the rumen in the MN fraction increasing the N out flow above N inflow.

NRC 1989 and NRC 2001 Model Evaluation

The predicted RDP and RUP supplied and required as predicted by the NRC (1989) and the NRC (2001) along with the AP and MP values from the 1989 and 2001 models, respectively, are reported in Table 2-7. For the NRC 1989 model, the g/d of RDP required varies little over the four diets, but as a percentage of DMI it decreases for cows fed the high RUP diets due to the greater DMI for those cows. The RDP requirement as a percentage of DMI did not decrease in the high RUP diets for the NRC 2001 because the RDP requirement is based on observed DMI leading to an increase in g/d of RDP required. The RDP supply decreased for both the NRC 2001 and NRC 1989 models for cows fed the low RDP diets. Therefore, the RDP supply as a fraction of requirement increased with increasing RUP for the 1989 NRC model but decreased for the 2001 model. For the NRC 2001 model the two low RDP diets supplied RDP at the requirement, but Reynal and Broderick (2005) suggested that the NRC 2001 is under-predicting the RDP supplied, which would mean all of our diets were above the RDP requirement.

The RUP requirement as a percentage of DMI prediction for the NRC 1989 and 2001 models decreased as the RUP increased due to an increase in DMI for cows fed the high RUP diets. The NRC 2001 model prediction of the RUP required (g/d) decreases as RUP increased due to the RUP required being predicted, in part, by the RUP supply. Therefore, as the supply increases in the high RUP diets the requirement decreases.

The AP and the MP requirements as percentages of DMI both decreased as the RUP increased due to the increase in DMI for cows fed those diets. All diets were above

the AP requirement according to the NRC (1989) and the MP requirement according to the NRC (2001) with the Low RDP/Low RUP diet meeting the respective requirements for both models.

The NRC 1989 and NRC 2001 predictions of N flows from the rumen and rumen N balance are reported in Table 2-8. The residual analysis (Table 2-9) showed no effect of RDP or RUP on the ability of either model to predict N flows from the rumen or ruminal N balance. The observed N intake was used to evaluate both models. Therefore, differences in the rumen N balance between the models is due to their respective predictions of MN and RUP-N flow. The NRC 1989 predicted a negative rumen N balance for all four diets with higher RUP and lower RDP resulting in a lower rumen N balance (Table 2-8). In contrast, the NRC 2001 significantly over-predicted the rumen N balance by 171.8 g/d (Table 2-10). There was a significant effect of RDP and RUP on the NRC 1989 predictions of MN flow with MN flow increasing from 366.9 g/d for cows fed the low RDP/low RUP diets to 475.3 g/d for cows fed the high RDP/high RUP diets. The NRC 2001 significantly under-predicted MN flow with a mean bias of 152.5 g/d (Table 2-10), and there was no significant effect of RDP or RUP on the NRC 2001 predictions (Table 2-8). Reynal and Broderick (2005) also observed that the NRC (2001) under-estimated MN flow at the omasum across four levels of dietary RDP. The NRC 2001 assumes no net influx of blood urea-N into the rumen, which could be leading to the lower MN flow prediction compared with the NRC 1989 model. Urea-N transfer to the rumen can range from 171 g/d (Lapierre and Lobley, 2001) to 422 g/d (Dinh et al., submitted) in dairy cows, and data in steers indicates that 66% of urea-N that enters the rumen in incorporated into microbial protein (Archibeque et al., 2002). Therefore,

because the NRC 2001 model does not account for MN derived from recycled urea-N, especially when N is limiting microbial protein synthesis, it under-estimates MN flow. The NRC 2001 subsequently uses MN flow to predict the RUP requirement, which makes the accuracy of this prediction important.

CONCLUSIONS

Although the protein concentration of all diets was at or above the NRC (2001) requirements, altering ratios and concentrations of RDP and RUP affected N flow out of the rumen. Rumen microbes appeared to have insufficient N to optimize NDF and DM total tract digestion potentially due to the lower RAN concentration inhibiting the fiber degrading bacteria. In contrast to our hypothesis, addition of RUP to low RDP diets did not augment microbial N flow to achieve that seen in high RDP diets. As expected, increasing the dietary N concentration led to an increase in urinary N excretion, with RUP being excreted in the feces. The NRC 1989 model predicted MN flow, total N flow, and rumen N balance more accurately than the NRC 2001 model. The NRC 2001 under-prediction of MN flow from the rumen is reflective of the model not accounting for recycled urea-N providing N for microbial protein synthesis. The under-prediction of MN flow by the NRC 2001 model resulted in the over-predicted the rumen N balance potentially leading to inaccuracies in the RDP and RUP requirement predictions.

Table 2-1. Ingredient and chemical composition of diets

	Diets			
	Low RDP		High RDP	
Item	Low RUP	High RUP	Low RUP	High RUP
Corn silage[1]	29.3	29.3	28.8	28.8
Grass Hay[2]	21.2	21.6	21.2	20.2
Ground Corn	25.0	25.3	25.0	25.1
Corn Starch	5.0	-	4.4	2.0
Megalac®	3.7	3.6	4.1	3.3
Soypass®	-	7.7	-	7.6
Soybean meal, 48%	13.6	10.6	13.7	10.5
Urea	0.4	0.4	1.2	1.2
Vitamin and mineral[3]	1.8	1.5	1.6	1.5
Chemical composition, % of DM				
DM, %	58.4	58.2	58.5	58.3
CP	16.7	18.2	19.8	20.6
NDF	31.9	32.3	30.7	33.2
ADF	15.0	15.9	14.4	15.1
Lignin	1.6	0.8	1.9	1.9
Ash	6.3	6.1	6.2	6.0
RDP[4]	10.1	10.0	12.5	12.2
RUP[5]	5.6	8.1	5.6	7.9
NE_L, Mcal/kg[6]	1.7	1.7	1.7	1.7

[1]Contained 9.0% CP, 43.0% NDF, 23.1% ADF, 2.3% lignin, and 3.4% ash
[2]Contained 14.2% CP, 60.3% NDF, 30.1% ADF, 2.5% lignin, and 6.3% ash
[3]The low RUP diets mix contained 0.07% Ca, 4.4% P, 9.9% Mg, 1.5% Cl, 3.4% K, 14.1% Na, 4.4% S, 44.0 ppm Co, 440 ppm Cu, 700 ppm Mn, 18.4 ppm Se, 1256 ppm Zn, 210,000 IU/Kg Vitamin A, 53,000 IU/Kg Vitamin D, and 1775 IU/Kg Vitamin E. For the high RUP diets mix contained 0.05% Ca, 2.8% P, 5.4% Mg, 0.94% Cl, 0.85% K, 8.9% Na, 1.24% S, 25.0 ppm Co, 250 ppm Cu, 400 ppm Mn, 12.0 ppm Se, 715 ppm Zn, 132,000 IU/Kg Vitamin A, 33,000 IU/Kg Vitamin D, and 1052 IU/Kg Vitamin E.
[4]Values predicted from NRC (2001)
[5]Values predicted from NRC (2001)
[6]Values predicted from NRC (2001)

Table 2-2. Milk yield and composition as influenced by RDP and RUP

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
Milk Yield, kg/d	34.6[ab]	35.2[a]	33.5[b]	34.0[ab]	1.7	0.90	0.37	0.06
4% FCM, kg/d[2]	33.5[ab]	34.5[a]	33.1[ab]	32.7[b]	1.8	0.26	0.56	0.09
Milk fat								
%	3.81	3.88	3.91	3.81	0.24	0.40	0.92	0.91
g/d	1.31	1.36	1.31	1.28	0.09	0.25	0.80	0.28
Milk true protein								
%	2.92[b]	2.92[b]	2.93[b]	2.99[a]	0.06	0.19	0.11	0.03
g/d	1.01	1.03	0.98	1.01	0.04	0.74	0.19	0.28
Milk solids non-fat								
%	4.82	5.27	5.19	5.24	0.20	0.32	0.20	0.38
g/d	1.68	1.84	1.74	1.78	0.11	0.33	0.11	0.97
Milk yield/DMI	1.75[a]	1.61[b]	1.67[ab]	1.59[b]	0.07	0.48	0.01	0.20
BW, kg	650[b]	663[a]	661[ab]	653[ab]	19	0.03	0.62	0.83

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP

[2]4% FCM (kg/d) = 0.4 x milk yield (kg/d) + 15 x fat yield (kg/d) (NRC, 2001)

[a-b]Means is the same row with unlike superscripts differ ($P < 0.05$)

Table 2-3. Ruminal pH and VFA concentration as influenced by RDP and RUP

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
Ruminal pH	6.19	6.11	6.21	6.28	0.10	0.33	0.93	0.23
RAN, mg/dL[2]	4.81[b]	6.33[b]	9.91[a]	10.67[a]	0.69	0.51	0.08	<0.01
VFA, mM								
Acetate	60.1[b]	64.4[ab]	67.0[a]	63.4[ab]	2.2	0.05	0.86	0.14
Propionate	24.6[ab]	22.9[b]	26.5[a]	25.1[ab]	2.4	0.86	0.12	0.05
Isobutyrate	0.6	0.6	0.6	0.7	0.1	0.70	0.59	0.53
n-butyrate	19.2	21.7	20.3	19.8	1.1	0.19	0.38	0.71
Isovalerate	0.7	0.7	0.8	0.8	0.1	0.56	0.65	0.08
n-valerate	3.0	1.5	1.7	1.6	0.8	0.37	0.31	0.41
Total VFA, mM	108.3	111.9	117.0	111.4	4.7	0.19	0.77	0.25
Acetate:Propionate	2.5[b]	2.9[a]	2.7[b]	2.7[b]	0.2	<0.01	<0.01	0.56

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP

[2]RAN = rumen ammonia-N

[a-b]Means is the same row with unlike superscripts differ ($P < 0.05$)

Table 2-4. Intake and total tract digestibility as influenced by RDP and RUP

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
DMI, kg/d	19.86[b]	21.97[a]	20.08[b]	21.45[a]	0.70	0.34	<0.01	0.71
DMI, % of BW	3.06[b]	3.33[a]	3.06[b]	3.28[a]	0.13	0.73	<0.01	0.70
NDF intake, kg/d	6.21[b]	6.92[a]	6.13[b]	7.02[a]	0.26	0.58	<0.01	0.95
NDF intake, % of BW	0.96[b]	1.05[a]	0.93[b]	1.00[a]	0.04	0.24	<0.01	0.77
Apparent total tract digestibility, %								
DM	75.6[a]	71.7[b]	76.0[a]	77.0[a]	1.3	0.05	0.24	0.03
OM	55.7	50.6	56.6	58.2	2.5	0.20	0.52	0.11
CP	71.5[a]	67.5[c]	74.9[b]	75.6[b]	1.4	0.06	0.16	<0.01
NDF	59.4[ab]	55.3[b]	60.8[a]	64.5[a]	2.0	0.05	0.91	<0.01
Apparent ruminal digestibility, %								
DM	59.8	56.5	48.7	53.9	6.7	0.54	0.89	0.34
CP	57.4	60.3	61.2	61.8	7.0	0.87	0.80	0.71

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP

[a-c]Means is the same row with unlike superscripts differ ($P <$ 0.0

Table 2-5. Nitrogen balance variables as influenced by RDP and RUP

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
N intake, g/d	531.9^{c}	640.5^{bc}	639.0^{c}	709.3^{a}	2.0	0.14	<0.01	<0.01
Fecal N, g/d	147.9^{b}	194.4^{a}	155.9^{b}	170.5^{ab}	11.0	0.11	<0.01	0.40
Urine excretion, kg/d	17.1^{b}	19.4^{a}	19.5^{a}	20.9^{a}	1.3	0.58	0.04	0.03
Urinary N, g/d	169.7^{b}	243.0^{b}	250.9^{b}	299.2^{a}	13.8	0.24	<0.01	<0.01
Milk N, g/d[2]	168.0^{ab}	170.0^{a}	160.0^{b}	168.6^{ab}	6.5	0.28	0.13	0.15
N balance, g/d[3]	53.9	33.2	70.7	71.1	21.4	0.57	0.59	0.16
N efficiency, %[4]	40.9^{a}	31.8^{b}	35.9^{ab}	34.0^{ab}	2.8	0.15	0.04	0.57

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP

[2]Milk N = milk NH_3-N (g/d) + MUN (g/d) + (milk protein/6.25) (g/d)

[3]N balance = N intake (g/d) – (fecal N (g/d)+ milk N (g/d) + urinary N (g/d))

[4]N efficiency = [(milk N (g/d) + retained N (g/d))/intake N (g/d)]*100

[a-c]Means is the same row with unlike superscripts differ ($P < 0.05$)

Table 2-6. Total N flow and microbial N flow out of the rumen as effected by RDP and RUP

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
DM flow								
Liquid fraction[2], kg/d	2.3	3.2	2.5	2.9	0.4	0.46	0.06	0.77
Solid fraction[3], kg/d	10.3	9.3	10.2	9.9	1.7	0.85	0.70	0.87
Total, kg/d	12.6	12.4	12.7	12.8	1.9	0.96	0.98	0.91
Total N flow								
Liquid fraction[4], g/d	139.7	180.1	147.0	165.4	23.1	0.51	0.09	0.82
Solid fraction[5], g/d	503.9	510.3	557.8	587.9	96.5	0.90	0.85	0.50
Total N, g/d	643.6	690.4	704.8	753.3	105.5	0.99	0.65	0.56
Microbial N flow								
LAB-N[6], g/d	139.7	180.1	147.0	164.2	23.2	0.49	0.10	0.80
LAB-N, % of MN	38.2	38.9	34.3	37.0	4.3	0.77	0.63	0.42
SAB-N[7], g/d	279.9	263.3	311.5	312.5	56.4	0.87	0.87	0.47
SAB-N, % of MN	61.8	61.1	65.7	63.0	4.3	0.77	0.63	0.42
Total, g/d	419.6	443.4	458.5	476.7	66.0	0.96	0.74	0.57
Microbial N/total N, %	66.2	64.0	65.2	63.4	1.7	0.89	0.22	0.61
RUP-N, g/d	224.0	247.0	246.3	276.6	42.8	0.93	0.54	0.55
Rumen N balance, g/d	-111.7	-49.9	-65.8	-44.0	102.7	0.85	0.69	0.80

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP
[2]Flow of material in the supernatant produced from the 4640 x g centrifuge
[3]Flow of material remaining on the 2.0 mm, 1.0 mm, 0.5 mm, 0.25 mm sieves, and pellet produced from 500 x g centrifuge
[4]Flow of N associated with the liquid fraction
[5]Flow of N associated with the solid fraction
[6]LAB-N = liquid associated bacteria-N
[7]SAB-N = solid associated bacteria-N

Table 2-7. NRC 1989 and NRC 2001 predictions of RDP, RUP, absorbed protein, and metabolizable protein

	Diets			
	Low RDP		High RDP	
Predictions[1]	Low RUP	High RUP	Low RUP	High RUP
NRC, 1989				
RDP supplied, g/d	2151.1	2197.9	2555.1	2582.6
RDP supplied, % of DMI	10.4	10.3	12.6	12.3
RDP required, g/d	2100.0	2087.8	2048.7	2080.8
RDP required, % of DMI	10.8	9.6	10.2	9.7
RDP supplied, % of required	100.0	108.7	125.5	127.7
RUP supplied, g/d	1251.3	1817.9	1231.5	1744.6
RUP supplied, % of DMI	6.0	8.5	6.1	8.3
RUP required, g/d	1239.9	1217.9	1223.1	1209.8
RUP required, % of DMI	6.4	5.6	6.1	5.6
RUP supplied, % of required	98.1	153.9	100.9	147.7
AP supplied, % of DMI[2]	13.5	15.4	15.2	16.7
AP required, % of DMI	12.8	11.4	12.2	11.4
AP supplied, % of required	109.7	137.7	126.7	146.8
NRC, 2001				
RDP supplied, g/d	2174.1	2286.4	2632.5	2710.0
RDP supplied, % of DMI	11.0	10.4	13.1	12.6
RDP required, g/d	2086.9	2283.6	2128.6	2239.3
RDP required, % of DMI	10.5	10.4	10.6	10.4
RDP supplied, % of required	104.2	100.2	123.7	121.0
RUP supplied, g/d	1140.5	1762.2	1138.4	1687.7
RUP supplied, % of DMI	5.7	8.0	5.7	7.9
RUP required, g/d[3]	1051.5	949.2	1043.3	917.0
RUP required, % of DMI	5.4	4.4	5.2	4.3
RUP supplied, % of required	114.3	192.9	114.4	185.7
MP supplied, % of DMI[4]	11.1	13.1	11.0	13.0
MP required, % of DMI	10.8	10.0	10.7	9.9
MP supplied, % of required	104.0	132.6	104.5	131.8

[1]All predictions were based on observed DMI and milk production. Tabular values were used for RUP and RDP

[2]AP = absorbed protein as calculated by NRC, 1989 except microbial CP estimated as a fraction of RDP when diets were deficient in RDP based on NRC (1989) text. Microbial CP, g/d = 0.9 x (RDP + 0.15 CP intake).

[3]RUP requirement calculated with assumption of adequate RDP (i.e. RUP requirement does not compensate for estimated reduction in microbial protein due to inadequate RDP).

[4]MP = metabolizable protein as calculated by NRC, 2001.

Table 2-8. Model (NRC, 1989 and NRC, 2001) predictions of ruminal N flows and rumen N balance as affected by RDP and RUP concentration.

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Parameters[2]	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
NRC, 1989								
MN, g/d[3]	366.86	414.69	446.04	475.26	13.55	0.20	<0.01	<0.01
MN/N intake[4]	0.69	0.65	0.70	0.67	0.008	0.32	<0.01	0.01
RUP-N, g/d	191.65	299.12	195.43	285.08	8.34	0.09	<0.01	0.31
RUP-N/ N intake	0.36	0.47	0.31	0.40	0.004	0.32	<0.01	<0.01
Total N, g/d[5]	558.50	713.81	641.47	760.34	21.80	0.14	<0.01	<0.01
Total N/ N intake	1.05	1.11	1.01	1.08	0.01	0.75	<0.01	<0.01
Rumen N balance, g/d[6]	-26.60	-73.32	-2.51	-51.07	8.87	0.92	<0.01	0.02
NRC, 2001								
MN, g/d	283.82	310.21	289.50	304.55	8.55	0.21	<0.01	1.00
MN/N intake	0.54	0.49	0.45	0.43	0.007	0.03	<0.01	<0.01
RUP-N, g/d	182.49	281.95	182.14	270.04	9.75	0.36	<0.01	0.34
RUP-N/ N intake	0.34	0.44	0.29	0.38	0.006	0.98	<0.01	<0.01
Total N, g/d	466.31	592.16	471.64	574.59	18.08	0.27	<0.01	0.55
Total N/ N intake	0.88	0.92	0.74	0.81	0.01	0.16	<0.01	<0.01
Rumen N balance, g/d	65.59	48.33	167.32	134.68	8.88	0.40	0.01	<0.01

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP

[2]Treatment averages of model predictions.

[3]Microbial N flow, g/d

[4]N intake was calculated from the observed DMI and N concentration of the treatment diets

[5]Total N = microbial N + RUP-N

[6]Rumen N balance = N intake (g/d)– total N flow (g/d)

Table 2-9. Residual (observed – predicted) analysis of NRC, 1989 and NRC, 2001 predictions of ruminal N flow and N balance as affected by RDP and RUP.

	Diets							
	Low RDP		High RDP			*P*-value[1]		
Parameters[2]	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
NRC, 1989								
MN, g/d[3]	52.73	28.70	12.50	1.42	64.99	0.92	0.79	0.61
MN/N intake[4]	0.09	0.05	0.03	-0.01	0.11	0.97	0.75	0.56
RUP-N, g/d	32.33	-52.09	50.82	-8.46	43.47	0.78	0.12	0.48
RUP-N/ N intake	0.05	-0.07	0.08	-0.01	0.07	0.84	0.14	0.56
Total N, g/d[5]	85.06	-23.04	63.31	-7.04	105.14	0.86	0.41	0.98
Total N/ N intake	0.15	-0.02	0.11	-0.02	0.17	0.92	0.41	0.90
Rumen N balance, g/d[6]	-85.06	23.04	-63.31	7.04	104.59	0.86	0.41	0.98
NRC, 2001								
MN, g/d	135.76	133.17	169.04	172.13	65.06	0.96	1.00	0.58
MN/N intake	0.25	0.22	0.27	0.24	0.11	0.99	0.75	0.84
RUP-N, g/d	41.49	-34.93	64.10	6.58	43.01	0.83	0.14	0.47
RUP-N/ N intake	0.07	-0.04	0.10	0.01	0.07	0.90	0.16	0.58
Total N, g/d	177.25	98.25	233.14	178.71	104.84	0.91	0.53	0.52
Total N/ N intake	0.32	0.17	0.37	0.24	0.17	0.96	0.43	0.73
Rumen N balance, g/d	-177.25	-98.25	-233.14	-178.71	104.30	0.91	0.53	0.52

[1]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP

[2]Treatment averages of residuals (observed – predicted) calculated from observed data (Dinh et al., submitted) and NRC (1989) and NRC (2001) predictions of N flow rates out of the rumen. All NRC 1989 and 2001 model predictions were based on observed DMI and milk production. Tabular values were used for RUP and RDP.

[3]Microbial N flow, g/d

[4]N intake was calculated from the observed DMI and N concentration of the treatment diets

[5]Total N = microbial N + RUP-N

[6]Rumen N balance = N intake (g/d)– total N flow (g/d)

Table 2-10. Bias and error of NRC, 1989 and NRC, 2001 models in predicting ruminal N flow and N balance compared with observed.

					% of Variance		
Predictions[1]	Mean[2]	Mean Bias[3]	Linear Bias[4]	RMSPE[5]	Mean	Linear	Residual error
NRC, 1989							
MN, g/d[6]	425.71	23.84	604.76	160.52	2.20	2.34	95.45
MN/N intake[7]	0.68	0.04	0.0004	0.27	2.52	0.63	96.84
RUP-N, g/d	242.82	5.65	1997.54*	114.29	0.24	15.29	84.46
RUP-N/ N intake	0.38	0.01	0.005*	0.18	0.48	14.00	85.51
Total N, g/d[8]	668.53	29.48	3351.91	259.31	1.29	4.98	93.72
Total N/ N intake	1.06	0.06	0.008	0.43	1.65	4.61	93.73
Rumen N balance, g/d[9]	-38.38	-29.48	2376.20	259.31	1.29	3.53	95.17
NRC, 2001							
MN, g/d	297.02	152.53*	99.83	219.60	48.24	0.21	51.54
MN/N intake	0.48	0.24*	0.0001	0.36	46.63	0.11	53.27
RUP-N, g/d	229.15	19.31	1979.91*	115.72	2.79	14.79	82.43
RUP-N/ N intake	0.36	0.04	0.005*	0.19	3.53	14.19	82.28
Total N, g/d	526.17	171.84*	2194.04	308.89	30.95	2.30	66.75
Total N/ N intake	0.84	0.28*	0.007	0.51	30.19	2.62	67.19
Rumen N balance, g/d	103.98	-171.84*	1970.02	308.89	30.95	2.06	66.99

[1]All NRC 1989 and 2001 model predictions were based on observed DMI and milk production. Tabular values were used for RUP and RDP.
[2]Mean of model prediction
[3]Mean bias was calculated as the average of the residuals (observed – predicted)
[4]Linear bias was calculated as r^2 x ($RMSPE^2 - MB^2$) where r^2 was determined from the regression of residuals vs. model predicted
[5]Root mean square prediction error = sqrt[(Σ(residuals^2))/n]
[6]Microbial N flow, g/d
[7]N intake was calculated from the observed DMI and N concentration of the treatment diets
[8]Total N = microbial N + RUP-N
[9]Rumen N balance = N intake (g/d)– total N flow (g/d)
*Mean bias was deemed significant if it was different from zero at $P < 0.05$ and linear bias was deemed significant if the slope of the regression line of residuals vs. predicted was significant at $P < 0.05$.

CHAPTER 3

EFFECT OF DIETARY RUMEN UNDEGRADABLE AND RUMEN DEGRADABLE PROTEIN CONCENTRATION ON UREA-N RECYCLING IN MID-LACTATIONG COWS

ABSTRACT

This study was designed to quantify urea-N flow in lactating dairy cows and determine the relative role of the kidney and the rumen in urea-N recycling. Eight mid-lactation Holstein cows were assigned to a repeated 4 x 4 Latin square, balanced for carryover effects. The isoenergetic diets contained 16.7, 18.2, 19.8, and 20.6% crude protein (CP) as a percentage of dry matter (DM). Diets were arranged in a 2 x 2 Factorial with two levels of rumen degradable protein (RDP; 10.0 and 12.5% of ration DM) and two levels of rumen undegradable protein (RUP; 5.6 and 8.0% of ration DM). There was no effect ($P > 0.1$) of CP concentration on rate (g/d) of urea-N transferred to the gastrointestinal tract (GIT) from the blood or urea-N transfer from the GIT to the blood. High CP diets increased the proportion of GIT entry that was utilized for anabolic purposes, and decreased the proportion of GIT entry returning to the ornithine-urea cycle. There was no effect of RUP or RDP on the rate of urea synthesis, urea-N transfer to the GIT, urea-N transfer to the blood from the GIT, or urea-N utilized for anabolism. The plasma urea N (PUN; mg/dL) was lowest for the low RDP diets but low PUN did not decrease transfer (g/d) of urea-N to the GIT. Microbial N from recycled urea-N as a percentage of total microbial N flowing from the rumen was significantly higher for cows fed the low RDP diets. No changes were observed in ruminal urea transporter RNA (bUT-B2) expression. The rate of transfer of urea-N across the rumen wall appeared to be independent of rumen ammonia and PUN concentrations indicating that potentially urea is transferred to the GIT through the facilitated diffusion of saturated urea transporters as a way to regulate N flow to the GIT and decrease the energy expenditure associated with converting ammonia to urea.

INTRODUCTION

Ruminants are not unique in their ability to transfer blood urea-N to the gastrointestinal tract (GIT), but they are unique in their ability to utilize recycled urea-N. Rumen bacteria can utilize urea-N for microbial protein synthesis, which is an excellent protein source to support milk production in lactating dairy cows. To date, there is only one review article evaluating data on urea-N recycling in lactating dairy cows (Lapierre and Lobley, 2001) because this type of research was expensive and technically difficult to perform in large animals. However, with the development and refinement of the doubly-labeled [^{15}N]urea method for use in ruminants (Lobely et al., 2000) it is no longer as difficult to quantify urea-N recycling in large animals. Moreover, the expense of this procedure has been greatly reduced due to the development of a simple and repeatable method to convert urea to N_2 in a monomolecular reaction, such that the newly formed N_2 maintains the identity of the original urea molecule (Marini et al., 2006). Because N_2 is measured on the more sensitive isotope ratio mass spectrometer, as opposed to traditional mass spectrometer, much less tracer must be infused further decreasing the cost of this type of research.

The concentration of N in the diet, the forage to concentrate ratio of the diet, and the overall dry matter intake (DMI) affect the rate urea-N is recycled to the GIT and proportion utilized for anabolic purposes (Marini, and Van Amburgh, 2003, Huntington, 1989, and Sarraseca et al., 1998). However, it is unclear how form of protein (rumen degradable protein (RDP) versus rumen undegradable protein (RUP)) might affect urea-N recycling. Increasing the RDP concentration of the diet generally leads to increased in rumen ammonia (RAN) concentration, and since the protonized form of ammonia (NH_4^+) is not diffusible across the rumen wall (Hogan, 1961) it builds up in the rumen and

inhibits urease enzyme activity (Cheng and Wallace, 1979) resulting in an inhibition of urea-N transfer into the rumen (Kennedy and Milligan, 1978). Therefore, the current theory is that high RDP diets will lead to less urea-N recycling. Forage to concentrate ratio and feed intake have indirect effects on urea-N transfer to the rumen by altering the ruminal pH, the main factor that determines the ratio of NH_3: NH_4^+. A low RAN concentration due to low RDP can inhibit microbial growth (Sannes et al., 2002), and limit the ability of the cow to utilize recycled urea-N. Recycled urea-N not utilized by the rumen microbes returns to the plasma urea-N (PUN) pool, and as this pool increases the kidney clearance of PUN also increases (Marini and Van Amburgh, 2003).

Splice variants of UT-B urea transporters (bUT-B1 and bUT-B2) have recently been identified and characterized in bovine rumen epithelial tissue (Stewart et al., 2005). The authors reported that the bUT-B2 is the predominant variant found in the stratum granulosum, stratum spinosum, and stratum basale layers of the rumen wall. Both UT-B proteins exhibit bidirectional transport of urea. Inhibition of the transporter with phloretin decreased urea transport in Ussing chambers, indicating that these proteins could be involved in facilitated diffusion of urea across the rumen wall (Stewart et al., 2005). A western blot analysis using antibodies designed against human UT-B urea transporters showed no difference in UT-B abundance in rumen tissue as the N intake of lambs increased (Marini et al., 2004). However, changes in urea transporter expression in the rumen tissue of lactating dairy cows in response to dietary changes has not been evaluated.

The specific objectives of this study were to determine the effect of RDP and RUP on urea-N recycling and utilization for anabolic purposes, and to determine relative roles of the kidney and the rumen in regulating urea-N recycling, and finally to determine

if urea transporters in the rumen are a regulated element of this process. The hypothesis was cows on a low RDP diet would have an increased need for recycled urea-N to provide N to the rumen microbes, and therefore more urea-N would be transferred to the rumen. Additionally, increasing RUP in a low RDP diet could alleviate an RDP deficiency by facilitating even greater urea-N recycling and subsequently utilization of recycled urea-N by the rumen microbes.

MATERIALS AND METHODS

Cows and Diets

As reported in Dinh et al (submitted), eight ruminally fistulated Holstein cows from the USDA-ARS Beltsville, MD were divided into two groups based on days in milk (DIM). The experiment was designed as a repeated 4 x 4 Latin square with 21-d periods with sampling occurring during the last 2 days of each period. The experiment was conducted with the approval of the University of Maryland Animal Care and Use Committee and the USDA Animal Care and Use Committee. Diet composition and marker explanation is reported in Dinh et al. (submitted).

Sample collection

Blood was sampled from the coccygeal vein every 4 h over the last 24 h of each period. Vacutainer tubes containing heparin and a 20 G needle were used to collect the samples. The samples were immediately centrifuged at 2060 x g for 15 minutes to separate the plasma, which was then transferred to microcentrifuge tubes and frozen. Urine and fecal samples were taken according to Dinh et al. (submitted) and grab samples of urine and feces were collected prior to the start of the [$^{15}N^{15}N$]urea infusion for determination of background concentrations of ^{15}N, [$^{15}N^{15}N$]urea, and [$^{15}N^{14}N$]urea. Milk samples were also taken and analyzed according to Dinh et al. (submitted).

Ammonia analysis

Ammonia was analyzed in milk, blood, and urine according to Dinh et al. (submitted). However, the acidified urine had to be diluted 10x with 0.05% acetic acid before the ammonia concentration could be determined. The blood was deproteinized before the ammonia analysis by combining 500 μL of plasma, 250 μL of distilled water, and 250 μL of 50% Trichoroacetic acid to a 1.5 mL eppendorf. Samples were vortexed and centrifuged at 7000 x g for 10 minutes (Force 712 microcentrifuge). Supernatant was recovered, diluted 4x with water, and analyzed. Milk ammonia was analyzed in the same manner as blood ammonia except that the milk was centrifuged at 7000 x g for 10 minutes and the cream was removed before the deproteinization procedure.

Urea and [^{15}N^{15}N]urea analysis

Concentrations of urea-N in blood and urine were determined by the Berthelot reaction. Blood was diluted 50:1 and urine 2000:1 prior to analysis. On a 96-well plate 25 μL of sample and 100 μL of urease (0.1 U/μL) were added to each well, shaken, and allowed to react for 20 minutes. Subsequently, 100 μL of phenol-nitroprusside-tartrate and 100 μL of alkaline hypochlorite were added to each well. The plate was shaken, incubated for 30 minutes prior to spectrophotometric measurements (Sunrise, Phenix Research Products, Hayward, CA) at 570 nm. The urea-N concentration was calculated by regression from a standard curve.

For analysis of [^{15}N^{15}N]urea and [^{15}N^{14}N]urea enrichment, urinary urea was isolated by aliquoting a volume of urine containing 30 μmol of urea into 5 mL test tubes. Two mL of nanopure water was added to each tube and the mixture was passed through ion exchange columns. The columns (Bio-Rad Laboratories, Richmond, CA) were prepared with 1.8 mL of resin (AG 50W-X8, 100-200 mesh H+, Bio-Rad Laboratories,

Richmond, CA). The column was rinsed with 10 mL of 1 M NaOH, followed by 2x 10 mL of nanopure water, 2x 10 mL of 1 M HCl, and rinsed with nanopure water until the effluent had a neutral pH. The columns were covered with 2 mL of nanopure water and allowed to sit overnight.

After the columns were prepared, the samples in the 5 mL tubes were applied to the columns and the flow-thru was discarded. Then the column was washed twice with 2.5 mL of nanopure water and the eluted 5 mL was saved in 15 mL screw cap containers. The samples were analyzed for urea and ammonia as described previously to determine the urea concentration and to verify that the all the ammonia had been removed. Once the urea concentration was determined, a sample equivalent to 3 μmol of urea was transferred to a 12 mL exetainer test tube (Labco Limited, High Wycombe, UK) with 13mm Hungate stoppers (Bellco Glass, Inc., Vineland, NJ) brought to 4 mL volume with nanopure water, and frozen.

The procedure for the monomolecular reaction of converting urea to N_2 gas was adapted from Marini et al. (2006). The 4 mL purified urea samples were allowed to thaw at room temperature and then bubbled with He for 20 minutes. The tubes were recapped and placed in liquid N. Once the sample was frozen 300 μL of NaOBr, which was bubbled with He for 20 minutes prior to use and continuously during the analysis, was added using an 22.5 gauge needle with a 1mL syringe. Once the NaOBr was frozen the tubes were gassed with He for 18 minutes using a 22.5 gauge needle attached to the He inflow and a second 22.5 gauge needle to allow gas to flow out of the tube. The outflow needle was removed first to create positive pressure inside the tube. The samples were allowed to thaw at room temperature then incubated in a 60°C water bath for 20 minutes. Even with the stringent conditions employed the reaction is not completely

monomolecular (both N atoms in the newly formed N_2 gas derived from a single urea molecule) resulting in an under-prediction of [$^{15}N^{15}N$]urea and an over-prediction of [$^{15}N^{14}N$]urea. Therefore, standards (0.0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% atom percent excess (APE)) made from the [$^{15}N^{15}N$]urea (98 atom % $^{15}N^{15}N$) used in the infusion were analyzed along with the samples to correct for the non-monomolecular reactions that occurred. The samples were then sent to the North Carolina State University Soil Sciences Department (Raleigh, NC) for analysis of the ratio of $^{15}N^{15}N$ to $^{14}N^{14}N$ (30/28) and $^{15}N^{14}N$ to $^{14}N^{14}N$ (29/28) gas through isotope ratio mass spectrometry. The ratios were then converted to atom percent according to the following equations:

Atom % $^{15}N^{14}N = [(29/28)/2]/(1 + 29/28 + 30/28)$

Atom % $^{15}N^{15}N = (30/28)/(1 + 29/28 + 30/28)$

The atom % for the samples taken pre-infusion were calculated in the same manner and were subtracted from atom % values from the samples taken after the start of the infusion to determine $^{15}N^{14}N$-APE and $^{15}N^{15}N$-APE. A correction factor (1.64%) for the non-monomolecular aspect of the reaction was determined from the standards and used to adjust the $^{15}N^{14}N$-APE and $^{15}N^{15}N$-APE values according to Marini et al. (2006).

Real-time PCR

Rumen papillae (0.5 g) were biopsied from the ventral caudal region of the rumen at the end of each period. The samples were rinsed with sterile saline and placed in 2.5 mL of RNAlater® (Ambion, Austin, TX) and stored in at 20°C overnight. The following day papillae were patted dry and transferred to nuclease free tubes for storage at –80°C. The RNA was isolated from each sample using the RNeasy Midi Kit with the on-column DNAse digestion (Qiagen Inc., Valencia, CA), and an additional centrifugation with 2.5 mL of RPE buffer at 3000 x g at room temperature to ensure that excess ethanol was

removed by transferring the column to a nuclease free 15 mL conical tube (not supplied in the kit). RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (Nanotech Technologies, Wilmington, DE). Quality of the RNA was assessed using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA).

Reverse transcription was conducted using the iScript cDNA Synthesis kit (Bio-rad Laboratories, Hercules, CA) with negative controls where no reverse transcriptase was added. Primers were designed using the bovine ESTs from GenBank and the Primer3 program (Table 3-1). The optimal annealing temperature was determined to be 56.8°C based on an annealing temperature gradient test (60.0°C, 59.7°C, 59.1°C, 58.1°C, 56.8°C, 56.0°C, 55.4°C, and 55.0°C) run during the real-time PCR reaction. Real-time PCR was performed in the Bio-Rad iCycler iQ detection system (Bio-Rad Laboratories) with 2 μL of the cDNA product, 12.5 μL of iQ SYBR Green Supermix (Bio-Rad Laboratories), 2 μL of the forward primer, and 2 μL of reverse primer in a 25 μL real-time PCR reaction volume. The following PCR conditions were employed: 95°C for 3 min, followed by 45 cycles of 94°C for 15 s, 56.8°C for 30 s, and 72°C for 30 s. Amplified real-time PCR products were visualized using a 2% agarose gel containing ethidium bromide to confirm that one product of the expected size was being amplified, and then purified using QIAquick Gel Extraction kit (Qiagen). The identity of the PCR amplification product was confirmed by sequencing using a CEQ8000 automated sequencer and DTCS Quickstart chemistry (Beckman Coulter, Inc., Fullerton, CA). The purified PCR products were serial diluted to generate standards (10^2 – 10^7 transcripts), which were used to generate a standard curve through quantitative analysis through real-time PCR. The standards were deemed acceptable if the standard curve had a correlation

greater than 0.995 and a PCR efficiency of 85 to 115%. The real-time PCR results for each sample were compared to the standard curve and starting quantities were reported.

Calculations

Clearance rate (CR): Clearance rate of urea by the kidney (L of blood per day per kg body weight) was calculated according to Kohn et al. (2005) using the flowing equation:

CR = UUN/PUN/BW

Where CR = kidney clearance rate, UUN = urinary urea-N (g/d), PUN = plasma urea N (g/L), and BW = body weight (kg). For clearance of urea by the mammary gland the equation was the following:

CR = MUN/PUN/BW where MUN = milk urea N (g/d).

Clearance rate of urea by the GIT was calculated according to the following equation:

CR = GER/PUN/BW where GER = urea-N transferred to the GIT (g/d).

Clearance rate of ammonia by the kidney and the mammary gland was calculated in the same manner. The transfer rates (g/d) were calculated by multiplying the clearance rates by the PUN (g/L).

Urea Recycling: Urea recycling was calculated according to Lobley et al. (2000) model (Figure 3-1) with the following equations:

GER = UER – UUE

Where UER = urea-N entry rate (g/d; urea synthesis), and UUE = urinary urea-N elimination (g/d) calculated from dilution of [$^{15}N^{15}N$]urea in urine.

$$UER = \{(E_{D30}/E_{U30})-1\}D_{30}$$

Where E_{D30} (98%) and E_{U30} are the enrichments of [$^{15}N^{15}N$]urea in the dose and in the urine, respectively and D_{30} is the dose (~2 g/d).

$u = UUE/UER$

Where u = proportion of the dose eliminated in the urine.

$ROC = \rho UER$

Where ROC = return to the ornithine cycle (g/d) and $\rho = UUE_{29}/(UUE_{29} + UUE_{30})$.

$r = \rho/(1-u)$

Where r = fraction of GER going back to the ornithine cycle

$f = u(UFE^*)/[(1-u)(UUE_{29} + UUE_{30})]$

Where f = fraction of GER going into feces and UFE* = urea-^{15}N in fecal excretion (g/d).

$a = 1 - f - r$

Where a = fraction of GER being used for anabolic purposes.

Multiplying GER by a, f, and r will give values for UUA, UFE, and ROC, respectively. Because the microbial N pool had not reached steady state with respect to ^{15}N incorporation, a non-steady-state model was used to calculate the microbial N from blood urea. Microbial N from PUN recycled to the rumen (MNU) was calculated according to the following equations:

• g^{15}N in fast pellet/**•t = g** ^{15}N flowing into microbial pool – g ^{15}N flowing out of microbial pool

This was then rearranged to:

• g^{15}N in fast pellet/**•t + g** ^{15}N flowing out of microbial pool = g ^{15}N flowing into microbial pool

Where the • **g**^{15}N in fast pellet was determined from ^{15}N analysis of the fast pellet from each time point and the g ^{15}N flowing out of microbial pool was determined from ^{15}N analysis of the solid and liquid fractions leaving the rumen as described in Dinh et al.

(submitted). The g ^{15}N flowing into the microbial pool was then multiplied by 1/APE of ^{15}N in blood, which was assumed to be equal to the APE of ^{15}N measured in the urine.

Statistical analysis

Data were analyzed using the Mixed procedure of SAS (2002) according to the following models:

$$Y_{ijklm} = \mu + G_i + C(G_j) + P(G_k) + RDP_l + RUP_m + (RDP * RUP)_{lm} + e_{ijklm}$$

Where,

μ = overall mean, G_i = effect of group (i = 1, 2), $C(G_j)$ = random effect of cow within group (j = 1, 2, 3, 4), $P(G_k)$ = effect of period within group (k = 1, 2, 3, 4), RDP_l = effect of level of RDP (l = 1, 2), RUP_m = effect of level of RUP (m = 1, 2), $(RDP * RUP)_{lm}$ = effect of interaction of RDP and RUP, and e_{ijklm} = residual error, assumed to be normally distributed.

The effect of dietary CP concentration was evaluated according to the following model:

$$Y_{ijkl} = \mu + G_i + C(G_j) + P(G_k) + T_l + e_{ijkl}$$

Where,

μ = overall mean, G_i = effect of group (i = 1, 2), $C(G_j)$ = random effect of cow within group (j = 1, 2, 3, 4), $P(G_k)$ = effect of period within group (k = 1, 2, 3, 4), T_l = dietary crude protein effect (l = 1, 2, 3, 4), and e_{ijkl} = residual error, assumed to be normally distributed.

RESULTS AND DISCUSSION

Urea Pools and Clearance Rates

As the protein concentration of the diet increased there was an increase in urinary urea-N excretion ranging from 93.8 g/d for cows fed the low RDP/low RUP diet to the 171.5 g/d for cows fed the high RDP/high RUP diet (Table 3-2). However, an increase in urine volume, presumably due to an increase in water intake, resulted in no difference in

the proportion of urinary urea-N as a fraction of total urine N. Plasma urea N concentrations were higher for cows fed the high RDP diets ($P < 0.01$) and the high RUP diets ($P < 0.01$). Although there was an increase in PUN concentration as dietary N concentration increased, there was no subsequent increase in kidney clearance rate of urea-N (L of blood cleared per d per kg BW). However, the higher PUN concentration for cows fed the high RDP and high RUP diets was associated with an increased transfer rate (g/d) of urea-N from the blood to the urine through the kidney. In contrast, in heifers, Marini and Van Amburgh (2003) reported an increase in kidney clearance of urea (mL of blood cleared per minute) as dietary N concentration increased.

The MUN concentration, like the PUN concentration, was higher in cows fed the high RDP diets ($P < 0.01$) and tended to be higher in cows fed the high RUP diets ($P = 0.06$). The clearance of urea-N (L of blood cleared per d per kg BW) by the mammary gland was higher for cows fed the low RDP diets ($P = 0.05$), but the transfer rate (g/d) followed the same trend on kidney transfer rate and increased as PUN increased.

In comparison, the clearance rate of urea-N by the GIT was not affected by the dietary RDP or RUP concentration, however, numerically it was twice as high for cows fed the low RDP/low RUP diet compared with cows fed the high RDP/high RUP diet. The transfer rates (g/d) of urea-N from the blood to the GIT were not affected by the RDP or RUP concentration, which is in contrast to the kidney and mammary gland transfer rates. Numerically, the GIT was increasing its clearance rate (L of blood cleared per day per kg BW) to maintain a constant amount (g/d) of urea transfer, whereas the kidney and the mammary gland were not altering their clearance rates to maintain a constant transfer rate resulting in an increase in the transfer rate (g/d) as the PUN concentration increased. The implication regarding urea-N recycling is that clearing of

PUN by the kidney and the mammary gland will regulate the PUN concentration and the amount of PUN available for transfer to the GIT. However, the GIT can alter its clearance rate to maintain a constant supply of urea-N regardless of diet induced changes in PUN concentration potentially to prevent a futile cycle of blood urea-N to rumen ammonia-N back to blood urea-N from developing.

Ammonia Pools and Clearance Rates

There was no effect of RDP or RUP on plasma ammonia-N concentration (Table 3-3). The ornithine-urea cycle converts absorbed ammonia to urea and is the primary mechanism in the cow for detoxifying ammonia to maintain low blood ammonia concentrations (Meijer et al., 1985). We observed increasing mammary and kidney clearance rates and transfer rates as dietary RDP and RUP increased leading to greater milk ammonia-N and urinary ammonia-N concentrations. The liver utilizes two pathways to remove ammonia from the blood, the high capacity/low affinity ornithine-urea cycle in the periportal cells and the low capacity/high affinity glutamine synthesis in the perivenous cells. Ammonia excretion in urine and milk represents amino acid metabolism in those organs and not transfer from the blood.

Urea N Kinetics

Urea-N fluxes within the cow are reported in Table 3-4. There was no effect of RDP or RUP on the quantity of urea synthesis, urea-N entering the GIT, or ammonia-N transfer from the GIT to the ornithine-urea cycle. There was also no effect of RDP or RUP on urea-N utilized for anabolic purposes, which according to Lobley et al. (2000), represents urea-N used by the microbes. Other studies in lambs (Marini et al., 2004), heifers (Marini and Van Amburgh, 2003), and steers (Archibeque et al., 2001) have all

observed an effect of dietary N concentration on the rates of urea-N recycling from the blood to the GIT and from the GIT to the blood.

In our study, sample collection began 24 h after the start of infusion. In other studies, urine and fecal collection did not begin until 48 h after the start of infusion (Archibeque et al., 2001, Marini and Van Amburgh, 2003, Marini et al., 2004). The implication is that our data represents the urea-N transferred to the rumen and absorbed through the rumen wall into the blood as ammonia. Whereas the longer infusion time before sample collection in these other studies results in data that reflects ammonia absorption through the rumen wall as well as digestion and absorption of microbial N that was initially derived from recycled urea-N. According to the Lobley et al. (2001) calculations used here and elsewhere, a shorter infusion time will result in a lower estimate of urea-N return from the GIT to the blood and a higher estimate of urea-N used for anabolism.

Our results contradict the theory that increasing RAN concentration is inhibitory to urease enzyme activity (Cheng and Wallace, 1979) and urea-N entry into the rumen (Kennedy and Milligan, 1978). We increased RAN concentration (Dinh et al., submitted) independently with our treatments with no apparent effect on urea-N transfer to the rumen. However, the RAN concentration observed in our treatments were low (4.8 to 10.7 mg/dl), and potentially even our highly RAN concentration was too low to inhibit urease enzyme activity or urea-N entry into the rumen.

The flow rates observed in our study were nearly 10 fold greater than previously reported for heifers (Marini and Van Amburgh, 2003) and steers (Archibeque et al., 2001), and also greater than data reported for dairy cows in a review by Lapierre and Lobley (2001). The higher numbers were expected because lactating dairy cows have

higher N intake than steers or heifers, which would ultimately lead to higher N and urea-N pools and flows rates. However, transfer to the GIT ranged from 60 to 70% of N intake and is comparable to data reported in sheep where entry into the GIT was 77% of N intake (Sarraseca et al., 1998). Marini and Van Amburgh (2003) observed that 19 to 30% of urea-N was being transferred to the GIT of N intake in heifers, which while lower than our observations, showed a similar trend of the greatest transfer as a percent of N intake occurring in animals fed diets with the lowest N concentration.

There was no effect of RDP or RUP on the g/d of UFE. According to the Lobley et al. (2000) calculations, UFE should represent urea-N excreted in the feces as ammonia-N, however, our fecal samples were dried in a forced-air oven which would volatize much of the ammonia that was present. Therefore, we believe our UFE values are more representative of urea-N that recycled to the microbes in the lower GIT (large intestines and cecum), utilized, and excreted as microbial N.

Rumen microbial N derived from recycled urea-N (Table 3-5) accounted for 12.9% of microbial N flowing from the rumen for cows fed the high RDP diets and 20.0% for cows fed the low RDP diets. As a percentage of recycled urea-N entering the GIT 23.9% was incorporated into microbial N for cows fed the low RDP/low RUP diets and dropped to 15.0% for cows fed the high RDP/high RUP diets. These effects were the opposite of what was observed for the whole-gastrointestinal tract utilization for anabolic purposes. One reason for the discrepancy could be that, the Lobley et al. (2000) calculation of urea-N used for anabolism is determined by subtracting rumen ammonia-N transfer to the ornithine-urea cycle and urea-N excretion in feces from the transfer of urea-N to the total GIT, and does not directly estimate utilization of urea-N by the rumen microbes leading to an accumulation of error in this estimate. There is evidence that the

rumen epithelium metabolizes ammonia before it reaches the blood based on decreased appearance of ammonia in the blood compared to disappearance from the rumen (Abdoun et a., 2003). Because the Lobley et al. (2000) model does not account for this metabolism, it over-predicts urea-N utilization for anabolic purposes. Therefore the difference between MNU and what was calculated as used for anabolism according to Lobley et al. (2000) could be explained by rumen epithelium metabolism of ammonia. Additionally, urea-N utilized by microbes in the large intestine and cecum is excreted without having the opportunity to be digested and absorbed. Although the utilized for anabolism value should represent microbial protein synthesis, microbial protein synthesized in the large intestine and cecum does not provide the same benefit to the ruminant as rumen microbial protein, and should not be combined into the same value, as is currently done with this method. Marini and Van Amburgh (2003) reported no effect of dietary N concentration on the proportion of urea-N transferred to the GIT that was subsequently used for anabolic purposes, but there was a linear increase in the amount of urea-N returning to the ornithine-urea cycle. Sunny et al. (2007) reported a concentration-dependent, first-order process in lambs where an increase in urea infusion into the blood led to an increase in the proportion of recycled urea-N returning to the blood and a decrease in the proportion that was captured for use by the rumen microbes. Our data of MNU versus the urea-N used for anabolism from the Lobley et al. (2000) calculation along with conflicting data from Marini and Van Amburgh (2003) and Sunny et al. (2007) highlight the need to elucidate urea-N transfer to the rumen and utilization by rumen microbes from transfer to the lower GIT.

Urea Transporters

Primers were designed to detect the UT-A, UT-B1, and UT-B2 urea transporters where UT-B1 and UT-B2 are splice variants of the SLC14a1 gene with UT-B2 (Stewart et al., 2005). Although we were able to detect UT-A (SCL14a2 gene) in kidney tissue collected from a previous experiment, we were unable to detect it in the rumen papillae tissue collected during this experiment. We were able to detect the UT-B2 transporter in rumen papillae. However, the starting quantities of RNA transcript in samples subjected to primers designed to detect only UT-B2 variant did not differ from samples subjected to primers designed to detect both the UT-B1 and UT-B2 variants, which contrasts with Stewart et al. (2005) where both the UT-B1 and UT-B2 variants were detected. Based on our limited sampling (once at the end of each period) the starting quantity of RNA of the UT-B2 transporter was not affected by the dietary concentration of RDP or RUP (Figure 3-1). Currently no data are available as to whether the time of day or time relative to feeding and/or milking impacts urea transporter expression. Similarly, Marini et al. (2004) also found no effect of dietary N concentration on urea transporter (UT-B) expression in lambs. However, previous studies (Marini and Van Amburgh, 2003; Ritzhaupt et al., 1997; Stewart et al., 2005) have reported a decrease in urea transporter expression with increasing dietary N concentration. The Marini and Van Amburgh (2003) data is not replicated so differences could be a result of variation between animals. The Ritzhaupt et al. (1997) and the Stewart et al. (2005) experiments were performed on isolated rumen tissue from steers using Ussing chambers where leaking from one side to the other can occur. In vivo, where there is greater urea-N concentration in the blood relative to the rumen it is likely that urea transporters transfer urea from the blood to the rumen, but whether transporter abundance is affected by dietary conditions has yet to be

determined. Stewart et al. (2005) suggested that urea transporters transfer urea into the rumen by facilitated diffusion, and the constant expression of bUT-B2 urea transporters in spite of increasing PUN concentration, as seen in our study, may be a way to maintain a constant amount of urea transfer to the rumen and regulate urea-N recycling.

CONCLUSIONS

The GIT is able to alter its clearance rate (L of blood cleared per d per kg BW) to maintain a constant transfer rate (g/d) of urea-N from the blood the GIT regardless of the clearance rate of the kidney or the mammary gland or increasing PUN and RAN concentrations. This regulation may be in part due to the facilitated diffusion of urea-N into the rumen via urea transporters, which move urea from the blood to the GIT. This limited transport would serve as a way to maintain a minimal supply of urea-N to the GIT when it is lacking in the rumen. This mechanism would also decrease the energy expenditure associated with recycling a greater amount of urea-N to the gut on high-protein diets and requiring reconversion of ammonia to urea-N via the ornithine-urea cycle. As a proportion of urea-N transferred to the GIT, cows fed the low RDP diets had less utilization of recycled urea-N for anabolic purposes and greater transfer to the blood according to the Lobely et al. (2000) calculations. In contrast, when microbial protein derived from recycled urea-N was calculated directly by measuring the tracer incorporation into the rumen microbes, cows fed the low RDP diets had the greatest utilization. Therefore, it is important to directly measure rumen microbial utilization of recycled urea-N since the values calculated using the Lobely et al. (2000) method may be masking utilization of recycled urea-N in the rumen.

Table 3-1. Primers used to detect bUT-A, bUT-B1, and bUT-B2 expression in the rumen.

Gene	Protein	Fragment size (bp)	Forward primer (5'⟶ 3')	Reverse primer (5' ⟶ 3')	Annealing Temperature (^{o}C)
SLC14a2	UT-A	137	GCCAAAGCCTGAAAGAGATG	GTGTGCATCCTGGGAGTTG	60.0
SLC14a1	UT- B1 /UT – B2	126	AATTCCCAGACAAGCCATTG	AGCGTGAAGGCTGTTCTGTT	56.8
SLC14a1	UT – B2	110	AGTGGACGGTCTTTGATTGG	ATCCAGGGGAAGACTCTGTG	56.8

Table 3-2. Urea pools and kidney, GIT, and mammary urea clearance rates as influenced by rumen degradable protein and rumen undegradable protein

	Diets							
	Low RDP[1]		High RDP[2]			*P*-value[3]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
Urea pools								
PUN, mg/dl[4]	9.0[c]	13.4[b]	14.0[b]	18.5[a]	1.1	0.92	<0.01	<0.01
MUN, mg/dl[5]	14.1[b]	16.2[b]	17.5[ab]	20.2[a]	1.3	0.76	0.06	<0.01
Urinary urea N, g/L	5.6[c]	6.8[b]	6.9[b]	8.2[a]	0.3	0.86	<0.01	<0.01
Urinary urea-N/Urine N, %	55.5	54.3	53.3	57.3	2.4	0.28	0.56	0.87
Clearance rates, L/d/BW^{-1}								
Kidney[6]	1.66	1.54	1.50	1.50	0.10	0.37	0.40	0.15
GIT[7]	7.97	4.64	4.59	3.76	1.51	0.41	0.18	0.18
Mammary[8]	0.08[a]	0.07[ab]	0.06[b]	0.06[b]	0.01	0.36	0.15	0.05
Transfer rates[9], g/d								
Kidney	0.15	0.20	0.20	0.27	0.01	0.58	<0.01	<0.01
GIT[10]	0.65	0.68	0.64	0.70	0.13	0.88	0.75	0.99
Mammary	0.007	0.009	0.009	0.01	0.0007	0.80	0.06	0.03

[1]RDP = rumen degradable protein
[2]RUP = rumen undegradable protein
[3]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP
[4]PUN = plasma urea-N
[5]MUN = milk urea-N
[6]Kidney clearance rate = [urine urea N (g/d)/PUN (g/L)]/BW (kg)
[7]GIT clearance rate = [GER (g/d)/PUN (g/L)]/BW (kg)
[8]Mammary clearance rate = [MUN (g/d)/PUN (g/L)]/BW (kg)
[9]Transfer rates calculated as PUN (g/L) x clearance rate (L/d BW^{-1})
[10]GIT = gastrointestinal tract
[a-b]LSmeans is the same row with unlike superscripts differ ($P < 0.05$)

Table 3-3. Ammonia pools and kidney, GIT, and mammary urea clearance rates as influenced by rumen degradable and rumen undegradable

	Diets							
	Low RDP[1]		High RDP[2]			*P*-value[3]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
Urea pools								
Urinary NH_3, g/d	0.20^{b}	0.28^{b}	0.32^{ab}	0.43^{a}	0.07	0.74	0.05	<0.01
PAN[4], mg/dl	0.36	0.37	0.38	0.36	0.02	0.47	0.74	0.74
Milk NH_3, mg/dl	0.10^{b}	0.13^{ab}	0.14^{a}	0.15^{a}	0.03	0.61	0.04	<0.01
Clearance rates, L/d/BW^{-1}								
Kidney[5]	0.10^{b}	0.12^{b}	0.13^{ab}	0.19^{a}	0.03	0.51	0.07	0.03
Mammary[6]	0.04^{b}	0.06^{ab}	0.06^{ab}	0.07^{a}	0.01	0.56	0.12	0.06
Transfer rates[7], g/d								
Kidney	$3.59x10^{-4}$	$4.19x10^{-4}$	$4.89x10^{-4}$	$7.08x10^{-4}$	$1.15x10^{-4}$	0.26	0.06	<0.01
Mammary	$1.58x10^{-4}$	$1.96x10^{-4}$	$2.09\ x10^{-4}$	$2.35x10^{-4}$	$4.90x10^{-5}$	0.74	0.08	0.02

[1]RDP = rumen degradable protein
[2]RUP = rumen undegradable protein
[3]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP
[4]PAN = plasma ammonia-N
[5]Kidney clearance rate = [urine NH_3(g/d)/PAN (g/L)]/BW (kg)
[6]Mammary clearance rate = [Milk NH_3 (g/d)/PAN (g/L)]/BW (kg)
[7]Transfer rates calculated as PUN (g/L) x clearance rate (L/d BW^{-1})
[a-b]LSmeans is the same row with unlike superscripts differ ($P < 0.05$)

Table 3-4. Urea-N kinetics as influenced by rumen degradable and rumen undegradable protein

	Diets							
	Low RDP[1]		High RDP[2]			*P*-value[3]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
Urea kinetics, g urea-N/d								
UER[4]	507.9	580.4	546.1	598.7	76.4	0.89	0.40	0.71
GER[5]	410.8	437.4	412.7	427.3	76.5	0.93	0.78	0.96
UUE[6]	93.8a	132.3b	133.4b	171.5c	9.3	0.98	<0.01	<0.01
ROC[7]	131.9	131.8	101.4	112.7	24.6	0.80	0.81	0.30
UFE[8]	23.2	30.4	22.1	21.9	4.6	0.35	0.37	0.21
UUA[9]	255.3	274.1	289.3	292.6	51.2	0.87	0.82	0.61
Fractional transfers, %								
UER to urine, u	22.7	27.3	27.1	30.0	3.7	0.81	0.30	0.33
UER to GIT, (1-u)	77.3	72.8	72.9	70.0	3.7	0.81	0.30	0.33
GER to ROC, r	31.6[a]	31.0[ac]	25.4[b]	26.6[bc]	1.9	0.60	0.88	<0.01
GER to feces, f	6.2[ab]	7.5[a]	5.6[b]	4.9[b]	0.7	0.11	0.66	0.02
GER to UUA, a	62.1[b]	61.4[b]	69.0[a]	68.5[a]	2.1	0.94	0.78	<0.01
Enrichment 29[10]	0.070	0.049	0.043	0.039	.007	0.29	0.09	0.02
Enrichment 30[11]	0.208	0.174	0.198	0.167	0.035	0.74	0.21	0.59

[1]RDP = rumen degradable protein
[2]RUP = rumen undegradable protein
[3]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP
[4]UER = urea entry rate into blood
[5]GER = gastrointestinal entry rate of urea (transport of blood urea to gut)
[6]UUE = urinary urea-N excretion
[7]ROC = return to the ornithine cycle (blood urea-N that is converted to ammonia and reconverted to blood urea)
[8]UFE = urea excreted in the feces
[9]UUA = urea used for anabolism
[10]Enrichment 29 = enrichment of the [$^{15}N^{14}N$]urea in the urine
[11]Enrichment 30 = enrichment of the [$^{15}N^{15}N$]urea in the urine
[a-c]LSmeans is the same row with unlike superscripts differ ($P < 0.05$)

Table 3-5. Utilization of recycled urea-N by the rumen microbes as affected by rumen degradable protein and rumen undegradable protein

	Diets							
	Low RDP[1]		High RDP[2]			*P*-value[3]		
Item	Low RUP	High RUP	Low RUP	High RUP	SEM	RUP*RDP	RUP	RDP
MNU[4], g/d	88.7	91.3	67.4	60.0	20.3	0.88	0.81	0.14
MNU, % of MN[5] flow	19.6	20.3	13.7	12.0	0.02	0.89	0.82	<0.01
MNU, % of UER[6]	18.1	13.7	12.5	10.4	0.02	0.41	0.11	0.02
MNU, % of GER[7]	23.9	18.6	17.9	15.0	0.03	0.62	0.17	0.07

[1]RDP = rumen degradable protein
[2]RUP = rumen undegradable protein
[3]RUP*RDP = effect of the interaction of RDP and RUP, RUP = main effect of RUP, RDP = main effect of RDP
[4]MNU = rumen microbial N derived from PUN
[5]MN = microbial N
[6]UER = urea-N entry rate into blood
[7]GER = urea-N gastrointestinal entry rate

Figure 3-1. Starting quantity of bUT-B2 RNA transcripts as influenced by rumen degradable protein (RDP) and rumen undegradable protein (RUP). There was no significant effect of RUP, RDP or RDP*RUP on the level of bUT-B2 RNA transcript level ($P_{RDPxRUP} = 0.81$, $P_{RDP} = 0.74$, $P_{RUP} = 0.72$)

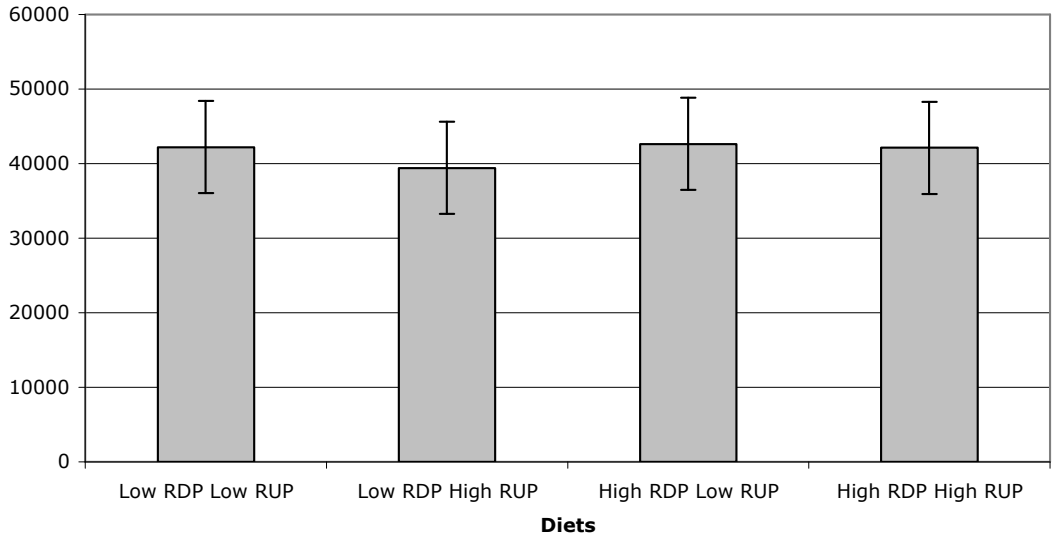
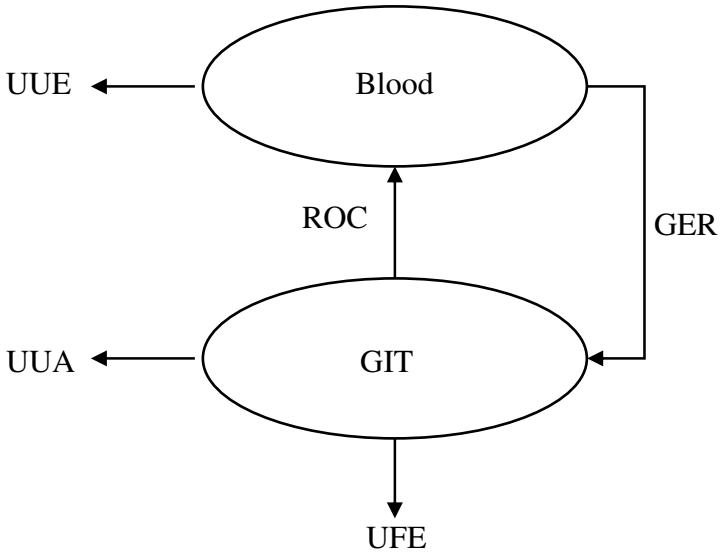

60000
50000
40000
30000
20000
10000
0
Low RDP Low RUP
Low RDP High RUP
High RDP Low RUP
High RDP High RUP
Diets

Figure 3-1. Model of urea-N recycling as reported in Lobley et al. (2001) where UUE = urinary urea-N excretion, ROC = urea-N returned to the ornithine cycle, GER = urea-N transferred to the gastrointestinal tract, UUA = urea-N utilized for anabolism, UFE = urea-N excreted in feces, GIT = gastrointestinal tract


UUE
Blood
ROC
GER
UUA
GIT
UFE

Literature Cited

Abdoun, K., F. Stumpff, and H. Martens. 2006. Ammonia and urea transport across the rumen epithelium: a review. Anim. Health Res. Rev. 7(1-17).

Abdoun, K., K. Wolf, G. Arndt, and H. Martens. 2003. Effect of ammonia on Na+ transport across isolated rumen epithelium of sheep is diet dependent. Br. J. Nutr. 90:751-758.

Allen, M. S. and D. R. Mertens. 1988. Evaluating constraints on fiber digestion by rumen microbes. J. Nutr. 118:261-270.

Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2001. Urea flux in beef steers: effects of forage species and nitrogen fertilization. J. Anim. Sci. 79:1937-1943.

Archibeque, S. L., J. C. Burns, and G. B. Huntington. 2002. Nitrogen metabolism of beef steers fed endophyte-free tall fescue hay: effects of ruminally protected methionine supplementation. J. Anim. Sci. 80:1344-1351.

Atasoglue, C., C. J. Newbold, and R. J. Wallace. 2001. Incorporation of [15N]ammonia by the cellulolytic ruminal bacteria Fibrobacter succinogenes BL2, Ruminococcus albus SY3, and Ruminococcus flavefaciens 17. Appl. Environ. Microbiol. 67:2819-2822.

Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63:64–75.

Bryant, M. P. and L. M. Robinson. 1963. Apparent incorporation of ammonia and amino acid carbon during growth of selected species of ruminal bacteria. J. Dairy Sci. 46:150-154.

Bunting, L. D., J. A. Boling, and C. T. MacKown. 1989a. Effect of dietary protein level on nitrogen metabolism in the growing bovine: I. Nitrogen recycling and intestinal protein supply in calves. J. Anim. Sci. 67:810-819.

Bunting, L. D., J. A. Boling, C. T. MacKown, and G. M. Davenport. 1989b. Effect of dietary protein level on nitrogen metabolism in the growing bovine: II. Diffusion into and utilization of endogenous urea nitrogen in the rumen. J. Anim. Sci. 67:820-826.

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

Cheng, K. J. and R. J. Wallace. 1979. The mechanism of passage or endogenous urea through the rumen wall and the role of ureolytic epithelial bacteria in the urea flux. Br. J. Nutr. 42:553-557.

Chou, C., J. M. Sands, J. Nonoguchi, and M. A. Knepper. 1990. Concentration dependence of urea and thiourea transport in rat inner medullary collecting duct. Am. J. Physiol. 258:F486-F494.

Conrad, H. R., J. W. Hibbs, and A. D. Pratt. 1960. Nitrogen metabolism in dairy cattle. Ohio Agricultural Experiment Station and Research Bulletin. 861. Wooster, OH.

Danley, M. M. and R. L. Vetter. 1973. Changes in carbohydrate and nitrogen fractions and digestibility of forages: maturity and ensiling. J. Anim. Sci. 37:994-999.

Dunlap, T. F., R. A. Kohn, L. W. Douglass, and R. A. Erdman. 2000. Diets deficient in rumen undegraded protein did not depress milk production. J. Dairy Sci. 83:1806-1812.

Ferrell, C. L., H. C. Freetly, A. L. Goetsch, and K. K. Kreikemeier. 2001. The effect of dietary nitrogen and protein on feed intake, nutrient digestibility, and nitrogen flux across the portal-drained viscera and liver of sheep consuming high-concentrate diets ad libitum. J. Anim. Sci. 79:1322-1328.

Garret, R. H., and C. M. Grisham. 1999. Biochemistry. Pp. 852-898. Harcourt, Inc., Orlando, FL.

Gillian, A. G. and J. M. Sands. 1992. Characteristics of osmolarity-stimulated urea transport in rat IMCD. Am. J. Phsyiol. 262:F1061-F1067.

Gillian, A. G., R. A. Star, and J. M. Sands. 1993. Osmolarity-stimulated urea transport in rat terminal IMCD: role of intracellular calcium. Am. J. Physiol. 265:F272-F277.

Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook. No 379. ARS-USDA, Washington, D.C.

Guyton, A. C. and J. E. Hall. 2000. Textbook of medical physiology. 10th ed. W. B. Saunders Company. Philadelphia, PA.

Hogan, J. P. 1961. The absorption of ammonia through the rumen of the sheep. Aust. J. Biol. Sci. 14:448-460.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press. New York, NY.

Huntington, G. B. 1986. Uptake and transport of nonprotein nitrogen by the ruminant gut. Fed. Proc. 45:2272-2276.

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

Huntington, G. B., E. J. Zetina, J. M. Whitt, and W. Potts. 1996. Effects of dietary concentrate level on nutrient absorption, liver metabolism, and urea kinetics of beef steers fed isonitrogenous and isoenergetic diets. J. Anim. Sci. 74:908-916.

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

Isozaki, T. J., J. W. Verlander, and J. M. Sands. 1993. Low protein diet alters urea transport and cell structure in a rat initial inner medullary collecting duct. J. Clin. Invest. 92:2448-2457.

Ivan, S. K., R. J. Grant, D. Weakley, and J. Beck. 2005. Comparison of a corn silage hybrid with high cell-wall content and digestibility with a hybrid of lower cell-wall content on performance in Holstein cows. J. Dairy Sci. 88:244-254.

Jackson, A. A., M. S. Daneilsen, and S. Boyes. 1993. A noninvasive method for measuring urea kinetics with a single does of [^{15}N^{15}N]urea in free-living humans. J. Nutr. 123:2129-2136.

Karkalas, J. 1985. An improved enzymatic method for the determination of native and modified starch. J. Sci. Food Agric. 36:1019-1027.

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

Kishore, B. K., J. Terris, P. Fernandez-Llama, and M. A. Knepper. 1997. Ultramicrodetermination of vasopressin-regulated urea transporter protein in microdissected renal tubules. Am. J. Physiol. 272:F531-F537.

Kohn, R. A., M. M. Dinneen, and E. Russek-Cohen. 2005. Using blood urea nitrogen to predict nitrogen excretion and efficiency of nitrogen utilization in cattle, sheep, goats, horses, pigs, and rats. J. Anim. Sci. 83(4):879-889.

Kohn, R. A., Z. Dou, J. D. Ferguson, and R. C. Boston. 1997. A sensitivity analysis of nitrogen losses from dairy farms. J. Environ. Manage. 50:417-428.

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

Levinsky, N. G., R. W. Berliner, and A. S. Preston. 1959. The role of urea in the urine concentrating mechanism. J. Clin. Invest. 38:741-748.

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

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

Lobley, G. E., P. J. M. Weijs, A. Connell, A. G. Calder, D. S. Brown, and E. Milne. 1996. The fate of absorbed and exogenous ammonia as influenced by forage or forage-concentrate diets in growing sheep. Br. J. Nutr. 76:231-248.

Marini, J. C. and M. S. Attene-Ramos. 2006. An improved analytical method for the determination of urea nitrogen isotopomers in biological samples utilizing continuous flow isotope ratio mass spectrometry. Rapid Comm. Mass Spec. 20:3736-3740.

Marini, J. C., J. D. Klein, J. M. Sands, and M. E. Van Amburgh. 2004. Effect of nitrogen intake on nitrogen recycling and urea transporter abundance in lambs. J. Anim. Sci. 82:1157-1164.

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

Meijer, A. J., C. Lof, I. C. Ramos, and A. J. Verhoeven. 1985. Control of ureagenesis. Eur. J. Biochem. 148:189-196.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds using refluxing in beakers or crucibles: Collaborative study. J. AOAC 85:1217–1240.

Moorby, J. M., and V. J. Theobald. 1999. Short communication: The effects of duodenal ammonia infusions on milk production and nitrogen balance of the dairy cow. J. Dairy Sci. 82:2440-2442.

Moscardini, S., T. C. Wright, P. H. Luimes, B. W. McBride, and P. Susmel. 1998. Effects of rumen-undegradable protein and feed intake on purine derivatives and urea nitrogen: comparison with predictions form the Cornell Net Carbohydrate and Protein System. 1998. J. Dairy Sci. 81:2421-2429.

National Research Council. 1989. Nutrient requirements of dairy cattle. 6th rev. ed. Natl. Acad. Sci., Washington, D. C.

National Research Council. 2001. Nutrient requirements of dairy cattle. 7th rev. ed. Natl. Acad. Sci., Washington, D. C.

National Research Council. 1985. Ruminant nitrogen usage. Natl. Acad. Sci., Washington, D. C.

Neilsen, S. and M. A. Knepper. 1993. Vasopressin activates collecting duct urea transporters and water channels by distinct physical processes. Am. J. Physiol. 265:F204-F213.

Nocek, J. E., J. H. Herbein, and C. E. Polan. 1980. Influence of ration physical form, ruminal degradable nitrogen and age on rumen epithelial propionate and acetate transport and some enzymatic activities. J. Nutr. 110:2355-2364.

Nolan, J. V. and R. A. Leng. 1972. Dynamic aspects of ammonia and urea metabolism in sheep. Br. J. Nutr. 27:177-194.

Oba, M., R. L. BaldwinVI, S. L. Owens, and B. J. Bequette. 2004. Urea synthesis by ruminal epithelial and duodenal mucosal cells from growing sheep. J. Dairy Sci. 87:1803-1805.

Olmos Colmenero, J. J., and G. A. Broderick. 2006a Effect of dietary crude protein concentration on ruminal nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 89:1694-1703.

Olmos Colmenero, J. J. and G. A. Broderick. 2006b. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. J. Dairy Sci. 89:1704-1712.

Peil, A. E., H. Stolte, and B. Schmidt-Nielsen. 1990. Uncoupling of glomerular and tubular regulations of urea excretion in rat. Am. J. Physiol. 258:F1666-F1674.

Perkin-Elmer. 1982. Analytical methods of atomic absorption spectrophotometry. Norwalk, CT.

Peterson, A. B. Estimation of rumen microbial protein production and ruminal protein degradation. Doctoral Disseration. University of Maryland. 2006.

Reynal, S.M. and G. A. Broderick. 2005. Effect of dietary level of rumen-degradable protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88:4045-4064.

Reynal. S. M., G. A. Broderick, and C. Bearzi. 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. J. Dairy Sci. 88:4065-4082.

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

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

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

Ritzhaupt, A., S. Wood, A. A. Jackson, B. J. Moran, and S. P. Shirazi-Beechey. 1998. Isolation of a RT-PCR fragment from human colon and sheep rumen RNA with nucleotide sequence similarity to human and rat urea transporter isoforms. Biochem. Soc. Trans. 26:S122.

Robinson, P. H. and R. E. McQueen. 1992. Influence of rumen fermentable neutral detergent fiber levels on feed intake and milk production of dairy cows. J. Dairy Sci. 75:520-532.

Roseler, D. K., J. D. Ferguson, C. J. Sniffen, and J. Herrema. 1993. Dietary protein degradability effects on plasma and milk urea nitrogen and milk nonprotein nitrogen in Holstein cows. J. Dairy Sci. 76:525-534.

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. ruminal fermentation. J. Anim. Sci. 70:3551-3561.

Sands, J. M., H. Nonoguchi, and M. A. Knepper. 1987. Vasopressin effects on urea and H_2O transport in inner medullary collecting duct subsegments. Am. J. Physiol. 253:F823-F832.

Sands, J. M., R. T. Timmer, and R. B Gunn. 1997. Urea transporters in kidney and erythrocytes. Am. J. Physiol. 273:F321-F339.

Sannes, R. A., M. A. Messman, and D. B. Vagnoni. 2002. Form of rumen-degradable carbohydrate and nitrogen on microbial protein synthesis and protein efficiency of dairy cows. J. Dairy Sci. 85:900-908.

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

SAS® User's Guide, Version 9.1 Edition 2002. SAS Inst. Inc., Cary, NC.

Satter, L. D. and L. L. Slyter. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr. 32:199-208.

Siddons, R. C., J. V. Nolan, D. E. Beever, and J. C. Macrae. 1985. Nitrogen digestion and metabolism in sheep consuming diets containing contrasting forms and levels of N. Br. J. Nutr. 54:175-187.

Shaver, R. D., L. D. Satter, and N. A. Jorgensen. 1988. Impact of forage fiber content on digestion and digesta passage in lactating dairy cows. J. Dairy Sci. 71:1556-1565.

Star, R. A., H. Nonoguchi, R. Balaban, and M. A. Knepper. 1988. Calcium and cyclic adenosine monophosphate as second messengers for vasopressin in the rat inner medullary collecting duct. J. Clin. Invest. 81:1879-1888.

Stewart, G. S., C. Graham, S. Cattell, T. P. 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(2):R605-R612.

St-Pierre, N. R. and C. S. Thraen. 1999. Animal grouping strategies, sources of variation, and economic factors affecting nutrient balance on dairy farms. J. Dairy Sci. 82(Supple. 2):72-83.

Sunny, N. E., S. L. Owens, R. L. Baldwin VI, 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. 85:1006-1013.

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

Uden, P., P. E. Colucci, and P. J. Van Soest. 1980. Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J Sci Food Agric. 31(7):625-632.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:2650-2661.

Weiss, W. P. and D. J. Wyatt. 2002. Effects of feeding dairy cows diets based on silage from corn hybrids that differed in concentration and in vitro digestibility of neutral detergent fiber. J. Dairy Sci. 85:3462-3469.

Wolf, R. R. 1981. Measurement of urea kinetics in vivo by means of a constant tracer infusion of di-^{15}N-urea. Am. J. Physiol. 240:E428-E434.

Yokoyama, M. T. and K. A. Johnson.1988. Microbiology of the rumen and intestines. Pages 125-144. *in* The ruminant animal; digestive physiology and nutrion. D. C. Church ed. Waveland Press, Inc., Prospect Heights, IL.

Zhang, C., J. M. Sands, and J. D. Klein. 2002. Vasopressin rapidly increases phosphorylation of UT-A1 urea transporter in rate IMCDs through PKA. Am. J. Physiol. 282:F85-F90.