Urea-N recycling in lactating dairy cows fed diets with 2 different levels of dietary crude protein and starch with or without monensin

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ABSTRACT

Rumensin (monensin; Elanco Animal Health, Greenfield, IN) has been shown to reduce ammonia production and microbial populations *in vitro*; thus, it would be assumed to reduce ruminal ammonia production and subsequent urea production and consequently affect urea recycling. The objective of this experiment was to determine the effects of 2 levels of dietary crude protein (CP) and 2 levels of starch, with and without Rumensin on urea-N recycling in lactating dairy cattle. Twelve lactating Holstein dairy cows (107 ± 21 d in milk, $647 \text{ kg} \pm 37 \text{ kg}$ of body weight) were fed diets characterized as having high (16.7%) or low (15.3%) CP with or without Rumensin, while dietary starch levels (23 vs. 29%) were varied between 2 feeding periods with at least 7 d of adaptation between measurements. Cows assigned to high or low protein and to Rumensin or no Rumensin remained on those treatments to avoid carryover effects. The diets consisted of approximately 40% corn silage, 20% alfalfa hay, and 40% concentrate mix specific to the treatment diets, with 0.5 kg of wheat straw added to the high starch diets to enhance effective fiber intake. The diets were formulated using Cornell Net Carbohydrate and Protein System (version 6.1), and the low-protein diets were formulated to be deficient for rumen ammonia to create conditions that should enhance the demand for urea recycling. The high-protein diets were formulated to be positive for both rumen ammonia and metabolizable protein. Rumen fluid, urine, feces, and milk samples were collected before and after a 72-h continuous jugular infusion of $^{15}\text{N}^{15}\text{N}$ -urea. Total urine and feces were collected during the urea infusions for N balance measurements. Milk yield and dry matter intake were improved in cows fed the higher level of dietary CP and by Rumensin. Ruminal ammonia and milk and plasma urea nitrogen concentrations corresponded to dietary CP concentration. As has been shown *in vitro*, Rumensin reduced rumen ammonia concentration by approximately 23% but did not affect urea entry rate

or gastrointestinal entry rate. Urea entry rate averaged approximately 57% of total N intake for cattle with and without Rumensin, and gastrointestinal rate was similar at 43 and 42% of N intake for cattle fed and not fed Rumensin, respectively. The cattle fed the high-protein diet had a 25% increase in urea entry rate and no effect of starch level was observed for any recycling parameters. Contrary to our hypothesis, Rumensin did not alter urea production and recycling.

Key words: urea recycling, Rumensin, nitrogen efficiency

INTRODUCTION

With the current environmental concerns regarding nitrogen (N) emissions and the potential effect on the environment (Jonker et al., 2002), improving N efficiency in dairy cattle is of interest (Colmenero and Broderick, 2006; Higgs et al., 2012). To enhance N efficiency, a better understanding of the amount and utilization of urea N recycling in dairy cows is important to improve the N efficiency of dairy cattle and thus reduce the environmental impact of dairy farms. Various studies have examined the effect of N intake, different forms of energy supplementation, and exogenous urea infusions on the quantity of urea recycled to the gastrointestinal tract (Ouellet et al., 2004; Valkeners et al., 2007).

Crude protein levels of approximately 14% appear to approach ruminal N deficiency in high-producing dairy cattle, with animals on these diets beginning to display impaired production and the resulting lower N efficiencies (Schwab et al., 2005; Recktenwald, 2007). An approach to improve N efficiency is to reduce urinary urea-N clearance by the kidney and stimulate urea-N re-entry to the rumen, to provide N for microbial maintenance and growth while creating ruminal conditions that enhance microbial uptake of the recycled urea N. Urea entry to the rumen has been hypothesized to be regulated via a nitrogen concentration gradient across the rumen wall in combination with alterations in the permeability of the epithelial boundary (Kennedy and Milligan, 1978; Abdoun et al., 2006) through changes in urea transporter activity (Marini et al., 2005). Nitrogen intake has been demonstrated to affect ruminal urea-

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N entry, with higher N intakes typically resulting in higher N recycling (Valkeners et al., 2007). However, the effect of N intake is not always consistent or linear (Reynolds and Kristensen, 2008). Further, urea-N can re-enter the rumen via saliva production during the process of chewing and rumination and can be a significant contributor to the urea-N pool (Maltby et al., 2005).

Dietary carbohydrate has been observed to stimulate urea-N entry to the rumen, most likely through effects on microbial growth and the subsequent depletion of ruminal N supply (Kennedy et al., 1981; Al-Dehneh et al., 1997). Carbohydrate fermentation might also have a secondary effect by altering the permeability of the epithelial boundary through VFA and CO₂ production (Abdoun et al., 2006). Both of these metabolic by-products have been demonstrated to stimulate urea-N entry and might aid in controlling N supply to the rumen to support the bacteria under conditions of high fermentable carbohydrate availability (Thorlacius et al., 1971; Rémond et al., 1993).

In vivo and in vitro data suggest that the ionophore Rumensin (Elanco Animal Health, Greenfield, IN) decreases feed proteolysis and rumen ammonia concentrations by reducing the obligate AA fermenting bacteria (Russell and Rychlik, 2001; Eschenlauer et al., 2002). Rumensin inhibits the metabolism and growth of mainly gram-positive bacterial species in addition to several strains of protozoa (Sylvester et al., 2009). In particular, its ability to slow the activity of certain hyper-ammonia-producing bacteria allows it to aid in conservation of feed AA and peptides, potentially enhancing feed AA flow out of the rumen (Choi et al., 2002; Eschenlauer et al., 2002). With that mechanism, more feed AA should escape rumen fermentation and increase the delivery of feed AA to the animal and also potentially increase the demand for recycled urea-N in the rumen because of lower ruminal ammonia concentrations. Russell and Strobel (1989) provided a general model of ionophore function in the rumen, which suggested that reduced ammonia production and altered fermentation should result in reduced urea formation and subsequently altered urea recycling and/or urea excretion. An indication that this model might translate to in vivo data was first described by Poos et al. (1979). However, the level to which this occurs in vivo in lactating cattle is unclear (Ruiz et al., 2001; Broderick, 2004).

Nitrogen intake, starch intake, and Rumensin all have potential effects on urea-N production and utilization along with potential changes in N efficiency. The objective of this study was to examine how those components work in conjunction to affect the animal's N metabolism, specifically ruminal N transamination

through urea production and metabolic fate. The hypotheses were that urea production would parallel N intake and be reduced by the addition of Rumensin because of a decrease in rumen ammonia production. Further, gastrointestinal urea-N entry would be enhanced by greater dietary starch intake, and the addition of Rumensin would increase the need for recycled urea-N due to lower ammonia production combined with greater microbial demand for ammonia N.

MATERIALS AND METHODS

Experimental Design

Twelve multiparous, lactating Holstein dairy cows [107 DIM $\pm$ 21 d, 647 kg of BW $\pm$ 37 kg, 3.0 $\pm$ 0.3 BCS (1–5 scale)] fitted with rumen fistulas were fed 1 of 8 different diets in a crossover design. Fistulas were established in the cattle approximately 2 mo before study. Animals were kept at the Cornell Teaching and Research Facility (Harford, NY), and the Cornell University Institutional Animal Care and Use Committee approved all animal-related procedures. The diets were formulated to contain either 16.5 or 15.3% CP and 29 or 23% starch, resulting in 4 compositionally different diets. In addition, Rumensin was included in the diets for 6 of the cows, and the other 6 received no Rumensin. Once assigned to Rumensin, the cows remained on that treatment to avoid long-term carryover effects due to potential long-term changes in rumen microbial population caused by the effects of the ionophore. Thus, with ionophore inclusion, 8 separate diets served as treatments for this experiment (Tables 1 and 2).

The cows were housed in individual tiestalls and fed a TMR once daily at approximately 0900 h at a 10% refusal rate. The TMR consisted of approximately 40% corn silage, 20% alfalfa hay, and 40% concentrate mix specific to dietary objectives (Tables 2 and 3). Wheat straw (0.5 kg/d) was included in the high-starch diets to enhance physically effective NDF and potentially reduce passage rate. Diets were formulated for 40 kg of ME- and MP-allowable milk using CNCPS v6.1 (Cornell University, Ithaca, NY; Fox et al., 2004; Tylutki et al., 2008) and were based on previous work that evaluated N efficiency in lactating cattle (Recktenwald, 2007).

The high-protein diets were formulated to be adequate for both MP and ruminal N balance, and the low-protein diets were formulated to be slightly deficient in ruminal N balance but to contain adequate MP. This was done to create a range in potential rumen fermentation conditions and ruminal N balance to evaluate ureagenesis and urea recycling. Starch levels were increased in the high-starch diets through the ad-

Table 1. The experimental design of the study¹

High protein (n = 6)				Low protein (n = 6)			
Rumensin (n = 3)		No Rumensin (n = 3)		Rumensin (n = 3)		No Rumensin (n = 3)	
High starch (n = 3)	Low starch (n = 3)	High starch (n = 3)	Low starch (n = 3)	High starch (n = 3)	Low starch (n = 3)	High starch (n = 3)	Low starch (n = 2)

¹Cows assigned to high or low protein and to Rumensin (Elanco Animal Health, Greenfield, IN) or no Rumensin remained on those treatments (fixed), but crossed on starch throughout the treatment period. Thus, for each starch treatment, 3 observations were made except for the low-protein, low-starch treatment without Rumensin, where 1 cow had unusually low ¹⁵N enrichment levels.

dition of steam-flaked corn, which should provide more fermentable starch for microbial growth and therefore increase ruminal N requirements for microbial growth and potentially enhance urea-N recycling (Table 2).

Rumensin was included in the TMR of 6 cows at a formulated rate of 400 mg/cow per day. To prevent any carryover effects of Rumensin, once a cow was assigned to the ionophore treatment, they remained on it for

the duration of the study. Cows were also maintained on either high- or low-protein diets to avoid potential long-term adjustments in ruminal and whole-body N status. Therefore, cows participated in 2 treatments each, in which they were switched between the 2 dietary starch levels. All cows were given bST per label (Posilac, Elanco Animal Health) to help maintain milk yield during the study period, and bST administration

Table 2. Treatment diets, ingredients, and chemical composition (%; DM basis)

Item	High protein, high starch	High protein, low starch	Low protein, high starch	Low protein, low starch
Corn silage	37.71	38.10	36.77	37.99
Alfalfa hay	18.69	19.10	18.43	19.42
Wheat straw	1.90	—	1.89	—
Steam-flaked corn	11.26	5.82	11.50	5.66
Cornmeal	10.92	5.78	11.37	5.49
Soybean meal	7.40	6.51	3.35	1.87
Citrus pulp	4.00	11.30	3.60	7.92
Soy hulls	—	4.02	1.72	7.45
Amino Plus ¹	3.79	4.17	5.45	6.73
Dextrose	0.83	—	0.90	—
Cargill fat	0.56	0.86	0.60	0.85
Blood meal	0.42	0.41	0.43	0.37
Sodium bicarbonate	0.42	0.43	0.43	0.43
Salt	0.40	0.39	0.39	0.40
Limestone	0.36	0.00	0.46	0.31
Monocalcium sulfate	0.36	0.33	0.33	0.27
Calcium sulfate	0.30	0.32	0.18	0.34
Urea	0.23	0.21	—	—
Magnesium oxide	0.20	0.18	0.16	0.16
Wheat middlings	0.08	1.92	1.76	4.17
Selenium ²	0.05	0.04	0.04	0.04
Alimet ³	0.03	0.03	0.03	0.03
1100 Dairy TM ⁴	0.03	0.03	0.03	0.03
Dairy ADE-AL/MA ⁵	0.03	0.02	0.02	0.03
Smartamine M ⁶	0.017	0.017	0.017	0.017
Zinc sulfate	0.004	0.004	0.004	0.004
Manganese sulfate	0.004	0.004	0.004	0.004
Dynamate/KMS 050 ⁷	—	—	0.12	—

¹Amino Plus (Ag Processing Inc., Hastings, NE) heat-treated soybean meal product, 51.5% CP, 1.30% fat, 2.18 Mcal of NE_L/kg.

²Cargill selenium (calcium 31% of DM, selenium 603 mg/kg; Cargill Inc., Minneapolis, MN).

³Alimet (DL-methionine hydroxy analog 88%; Novus Inc., St. Louis, MO).

⁴Cargill 1100 Dairy trace mineral (calcium 0.022%, sulfur 18.816%, copper 30,317 mg/kg, manganese 136,466 mg/kg, cobalt 3,393 mg/kg, iodine 3,040 mg/kg, zinc 153,916 mg/kg; Cargill Inc.)

⁵Cargill Dairy ADE-AL/MA (vitamin A, 30,464 IU/g, vitamin D, 5,862 IU/g, vitamin E, 93,784 IU/kg; Cargill Inc.).

⁶Smartamine M (Adisseo, Alpharetta, GA).

⁷Dynamate/KMS (sulfur 24%, magnesium 12%, and potassium 19%; Mosaic Co., Plymouth, MN).

Table 3. Measured chemical composition (% of DM unless otherwise noted) of the forages fed

Item	Corn silage	Alfalfa hay	Wheat straw
DM, %	31.9	88.2	88.9
CP	8.49	19.6	4.89
Soluble CP, % of CP	67.7	40.1	41.8
NDF	42.9	42.4	79.8
ADF	23.5	32.3	52.7
Lignin, % of NDF	6.59	14.7	12.2
Starch	31.9	1.48	1.57
Sugar	1.39	6.71	2.01
Crude fat	3.40	1.54	1.45
Neutral detergent insoluble CP, % of CP	10.7	18.8	31.5
Acid detergent insoluble CP, % of CP	3.77	4.31	16.3

was synchronized such that all cows were at the same stage of the bST cycle during stable isotope infusions. Before and after treatment, the BW and BCS of the cattle were measured and BCS was assessed on a 1 to 5 scale (Wildman et al., 1982).

The cattle were given an initial adaptation period of approximately 21 d to the fixed components of the diet and were given at least 7 d of adaptation to the starch diet before $^{15}\text{N}^{15}\text{N}$ urea infusions and related measurements were taken, with the average starch adaptation period being 17 d. Each period change was done on a cow-by-cow basis and not by treatment. Cows were moved to metabolism stalls for the infusion and collection periods. Cows were milked 3 times per day at 0700, 1500, and 2300 h in their stalls with a portable milking machine, and milk was weighed via a calibrated and certified digital scale. Each milking was sub-sampled in the milk pail and preserved with 2-bromo-2-nitropropane-1,3-diol for milk component analysis (Dairy One, Ithaca, NY). Feces, urine, plasma, and rumen fluid samples were taken before jugular $^{15}\text{N}^{15}\text{N}$ -urea infusions to establish ^{15}N background values. Before the infusion period, feces were collected by either voluntary elimination or rectal palpation into 1-gallon plastic bags and then frozen. Before $^{15}\text{N}^{15}\text{N}$ -urea infusions, urine was collected by manual agitation of the lower vulva, 50% H_2SO_4 (vol/vol) was added until pH <2, and then samples were frozen in conical vials. Blood was collected into Vacutainers containing heparin (Becton Dickinson, Rutherford, NJ) via the coccygeal vessels, placed on ice, and centrifuged at $1,500 \times g$ for 15 min to obtain plasma. Rumen contents were collected for ammonia ($\text{NH}_3\text{-N}$) measurements by sampling at approximately 4 to 5 locations throughout the rumen. The rumen samples were squeezed through 4 layers of cheesecloth, and the extracted fluid immediately acidified with 1 mL of 50% (vol/vol) H_2SO_4 in 40 mL of rumen fluid and frozen in conical vials for $\text{NH}_3\text{-N}$ analysis.

For the infusion period, animals were prepared with indwelling jugular vein catheters (Micro-Renathane polyvinyl; Braintree Scientific Inc., Braintree, MA) at

least 1 d before the start of a continuous infusion of $^{15}\text{N}^{15}\text{N}$ -urea (98% purity, Cambridge Isotope Laboratories Inc., Andover, MA) in sterile saline (9 g of NaCl/L) at 0.0278 g of urea/h. The labeled urea solution was prepared as needed and refrigerated at 4°C if not used immediately. The urea solution was infused using a MasterFlex Pump 4S (Cole-Parmer, Vernon Hills, IL) with an EasyLoad head and L/S 14 tubing and set for 0.22 to 0.28 mL/min infusion rate.

Feces, urine, plasma, and rumen fluid samples were taken at least 72 h after initiating the infusion using the same procedures as those used for background sampling, except that urine was collected via urinary catheters maintained for the purpose of total urine collection and feces were collected from trays mounted below the stalls. The infusion period was established based on the data of Lobley et al. (2000) and Marini and Van Amburgh (2003), indicating that a plateau in $^{15}\text{N}^{15}\text{N}$ urea enrichment within the animal would be achieved by that time. The urinary catheters were placed in the cows 24 h before the beginning of the infusion. The catheters were 8-gauge Foley catheters (22 French, 75-mL; C. R. Bard Inc., Covington, GA) and were connected to sealed plastic bags that were emptied 3 times a day into a carboy that contained adequate H_2SO_4 to reduce the pH to <2. For total collection, urine was composited every 24 h; the composite represented 10% of the total excretion weight for the period and was stored at 4°C until analyzed. Feces were subsampled on a daily basis and then combined over the collection period.

The analysis for fecal and plasma ^{15}N enrichment were determined on freeze-dried samples with a NC2500 elemental analyzer (Carlo Erba, Milan, Italy) interfaced to a Delta Plus isotope ratio mass spectrometer (ThermoFinnigan, Bremen, Germany). Urinary urea was isolated via a cation exchange resin (AG 50W-X8, 100–200 mesh hydrogen form, Bio-Rad Laboratories, Hercules, CA), diluted to 6 mmol/L, and reacted with lithium hypobromite under vacuum (Marini and Attene-Ramos, 2006). The resulting N_2 gas was ana-

lyzed with a PDZ Europa Geo 20/20 isotope ratio mass spectrometer (EA-IRMS; Sercon Ltd., Cheshire, UK) attached to the ANCA-trace gas/liquid system. The model of Lobley et al. (2000) was used to calculate urea kinetics.

Total N content of the wet feces was analyzed with a Kjeltec 2300 analyzer (Foss Analytical, Hillerød, Denmark). Ruminal ammonia and plasma urea concentrations were measured using the procedure of Chaney and Marbach (1962).

Feed ingredients, TMR, and Orts were sampled daily during both the adaptation and infusion periods, dried in a forced-air oven at 60°C for 24 h, and then subsampled for weekly determination of the chemical composition. The subsamples were ground through a Wiley mill (A. H. Thomas, Philadelphia, PA) with a 1-mm screen and another subsample was sent to Dairy One (Ithaca, NY) for wet chemistry analyses. Diet ingredients were analyzed for chemical components necessary to meet the inputs necessary for the CNCPS (wet chemistry package 315, Dairy One Forage Laboratory). The TMR and Orts were analyzed for DM, CP, soluble CP, NDF, ADF, lignin, starch, sugar, crude fat, neutral detergent insoluble CP and acid detergent insoluble CP (Ration Balancer Plus package, Dairy One Forage Laboratory).

Statistical Design and Analysis

Data were analyzed with the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with the model

$$Y_{ijklm} = \mu + T_i + P_j + S_k + R_l + PS_{jk} \\ + PR_{jl} + SR_{kl} + C_m + P_n + E_{ijklm},$$

where Y_{ijklm} = dependent variable, μ = overall mean, T_i = effect of dietary treatment i , P_j = effect of dietary CP level j , S_k = effect of dietary starch level k , R_l = effect of feed additive l (Rumensin vs. no additive), PS_{jk} = interaction of dietary CP level j and starch level k , PR_{jl} = interaction of dietary CP level j and feed additive l , SR_{kl} = interaction of dietary starch level k and feed additive l , C_m = effect of cow m , P_n = effect of experimental period n , and E_{ijklm} = residual error. All terms were considered fixed except for C_m and E_{ijklm} , which were considered random.

Repeated measures were used for analysis of daily milk yield, milk components (fat %, protein %, lactose %, and MUN) and component yield, DMI, and N intake. Autoregressive order 1 was used as the covariance structure with repeated measures. The LSMEANS option was used to determine treatment, factor, and factor interaction means, and these were adjusted using

the PDIFF option. Type 3 tests of the fixed effects were used to determine significance, which was declared at $P < 0.05$ and a statistical trend was declared at $P < 0.10$.

RESULTS AND DISCUSSION

Production Outcomes

All cattle finished the allocated treatments; however, one cow receiving the low-protein, low-starch diet without Rumensin had unusually low ^{15}N enrichment values (less than 50% of expected) after analysis of the infusion period data and was not included in the analysis of urea recycling for one period. The treatment means for BW of cattle ranged from 584 to 688 (± 37.3) kg and BCS ranged from 2.8 to 3.1 (± 0.29) with no significant differences among treatments for either BW or BCS. Overall, the cattle were approximately 107 DIM (± 21 d) during the first ^{15}N -urea infusion and 117 DIM (± 21 d) for the second infusion. No significant differences were observed among treatment means for DIM at initiation of infusions.

Actual dietary CP concentrations were close to formulated values for both the high- and low-protein diets (Tables 2 and 4). Dietary starch concentrations were also similar to formulated values. Alfalfa hay and corn silage were relatively consistent throughout the duration of the study, varying in CP concentration from 18.3 to 21.2% and from 8.3 to 8.9% CP, respectively, and this consistency resulted in little variance among treatment diets. Dry matter intake was significantly greater ($P < 0.01$) for cows consuming the high-protein diets (averaging 25.7 vs. 22.9 kg of DMI/d), and we observed a trend ($P = 0.06$) for DMI to be higher for Rumensin-fed cattle (24.9 vs. 24.1 kg of DMI/d; Table 5). The DMI observed on this study were similar to studies feeding high levels of corn silage in combination with alfalfa silage (Wattiaux and Karg, 2004) and lower protein diets (Law et al., 2009). Previous work has demonstrated DMI inhibition with low dietary CP concentrations, due mainly to effects of ruminal N deficiency on microbial fermentation (Ruiz et al., 2002; Broderick, 2003). In this study, because the low-protein diets were formulated to be near zero in rumen N balance, the observation of lower DMI indicates that the formulation objectives were most likely achieved.

Milk yield was significantly greater for cattle fed high-protein and high-starch diets (by 7.7 and 3.4%, respectively) and were consistent with the data of Law et al. (2009), where cattle fed diets ranging from 11.4 to 17.3% CP yielded between 25.4 and 35.4 kg of milk per day. Milk fat percentage and milk fat yield ranged from 3.58 to 4.01% (Table 5), were not different among treatments, and were similar to or higher than those

Table 4. Analyzed chemical composition (% of DM unless otherwise noted) of the treatment diets

Item	High protein, high starch	High protein, low starch	Low protein, high starch	Low protein, low starch
DM, %	53.0	55.0	55.9	55.6
CP	16.7	16.6	15.1	15.2
Soluble CP, % of CP	35.4	36.1	36.2	36.7
NDF	29.8	32.2	31.3	34.9
ADF	17.7	19.3	17.9	21.5
Lignin, % of NDF	9.9	9.5	10.2	9.3
Starch	27.5	23.0	29.9	21.0
Sugar	6.1	6.2	5.0	6.3
Crude fat	2.7	2.8	2.9	2.8
Neutral detergent insoluble CP, % of CP	9.3	11.9	15.6	15.5
Acid detergent insoluble CP, % of CP	2.0	2.7	2.2	3.0

reported in cattle fed similar diets with similar milk yields (Wattiaux and Karg, 2004). Milk protein percentage and yield were significantly greater for cattle fed the higher starch diets, consistent with the observation that higher starch yields greater propionate supply, which affects milk protein yield (Raggio et al., 2006).

Rumen pH, NH₃-N, MUN, and Plasma Urea N

Rumen pH was relatively consistent among all treatments, with dietary means ranging from 5.83 to 6.08, and no significant effect of dietary CP, starch, or Rumensin (Table 6). Milk urea-N, plasma urea-N (**PUN**), and ruminal NH₃-N concentrations were significantly increased by feeding the high-protein diets ($P < 0.01$; Tables 5 and 6), consistent with previous data on lac-

tating dairy cattle fed similar levels of CP (Broderick, 2003; Ipharraguerre and Clark, 2005).

Rumensin significantly decreased rumen NH₃-N concentrations ($P = 0.03$) among treatments (Figure 1; Table 6) and this was consistent with the model described by Russell and Strobel (1989) where in vitro bacterial ammonia production was reduced through a reduction in feed AA degradation. Although not significant, a numerical increase was observed in the PUN of cattle fed Rumensin. This paradoxical effect has been previously observed but the reason is unclear (Duffield et al., 2003; Martineau et al., 2007). Consistent with previous data, the effect might be indicative of a protein sparing effect of Rumensin, decreasing ruminal AA degradation with concomitant increases in postruminal N digestion in the small intestine, thereby

Table 5. Body weight, BCS, DIM, DMI, N intake, milk and milk composition and component yields and plasma and milk urea nitrogen content of dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin (Rum; Elanco Animal Health, Greenfield, IN)

Item	High protein				Low protein				SEM	<i>P</i> -value		
	Rumensin		No Rumensin		Rumensin		No Rumensin					
	High starch	Low starch	High starch	Low starch	High starch	Low starch	High starch	Low starch		Protein	Starch	Rum
No. of cows	3	3	3	3	3	3	3	2				
BW, kg	637	646	688	676	670	665	607	584	37.3	0.40	0.47	0.66
BCS	2.8	2.8	3.0	2.8	3.0	3.0	3.0	3.1	0.29	0.31	0.85	0.61
DIM	110	106	117	113	130	116	117	126	20.7	0.59	0.67	0.90
DMI, kg/d	26.8	26.1	24.3	24.9	23.2	22.7	22.6	22.1	1.47	<0.001	0.72	0.06
N intake, g of N/d	736	691	644	667	556	542	545	562	43	<0.001	0.84	0.14
CP of diet, % of DM	17.1	16.5	16.4	16.6	15.0	14.9	15.1	15.6	0.30	<0.001	0.87	0.61
Milk yield, kg/d	31.2	31.5	29.3	30.3	31.7	27	28.1	26.9	1.21	<0.05	<0.05	<0.1
Milk fat, %	3.97	4.01	3.86	3.79	3.74	3.58	3.71	3.83	0.35	0.57	0.96	0.95
Milk fat yield, kg/d	1.71	1.74	1.54	1.57	1.62	1.31	1.43	1.40	0.16	0.19	0.24	0.47
Milk protein, %	2.97	2.98	2.73	2.66	2.73	2.60	2.84	2.80	0.16	0.55	<0.01	0.70
Milk protein yield, kg/d	1.25	1.28	1.07	1.09	1.16	0.96	1.08	1.01	0.08	0.13	0.03	0.20
Milk lactose, %	4.99	5.01	4.94	4.99	4.81	4.86	4.60	4.57	0.10	<0.01	0.06	0.12
Milk lactose yield, kg/d	2.11	2.15	1.97	2.06	2.08	1.78	1.75	1.68	0.09	<0.01	0.14	0.05
Milk urea N, mg/dL	10.84	9.30	11.75	10.94	8.43	8.95	6.94	8.01	0.75	<0.01	0.34	0.98
Plasma urea N, mg/dL	15.26	12.49	13.47	13.28	8.17	8.75	7.67	6.83	0.97	<0.01	0.02	0.29

Table 6. Rumen pH, ammonia levels, and fecal and urinary nitrogen excretion for dairy cows supplemented with different dietary CP and starch concentrations, with or without Rumensin (Rum; Elanco Animal Health, Greenfield, IN)

Item	High protein				Low protein				SEM	<i>P</i> -value		
	Rumensin		No Rumensin		Rumensin		No Rumensin					
	High starch	Low starch	High starch	Low starch	High starch	Low starch	High starch	Low starch		Protein	Starch	Rum
No. of cows	3	3	3	3	3	3	3	2				
Ruminal NH ₃ -N, mg/dL	10.13	8.29	13.99	11.32	4.89	5.79	6.94	5.75	1.38	<0.01	0.16	0.03
Ruminal pH	6.08	5.96	5.91	5.83	6.04	5.94	5.93	6.03	0.11	0.63	0.29	0.40
N intake, g/d	738	698	641	673	563	551	552	562	41.4	<0.001	0.90	0.30
Fecal N, g/d	217	215	202	190	176	179	181	183	13.2	<0.01	0.80	0.40
Urinary N, g/d	229	183	208	199	175	142	125	153	18.8	<0.01	0.27	0.45
Urine urea N, g/d	124.0	97.1	111.8	114.1	87.1	83.0	71.9	72.6	12.8	<0.01	0.20	0.22
Milk N, g/d	193	203	165	173	180	153	173	155	14.3	0.16	0.22	0.36
N balance, g/d	99	97	66	111	32	77	73	71	33.0	0.24	0.38	0.87
N balance, %	13.5	13.8	8.8	16.9	5.5	4.9	13.1	4.3	4.94	0.50	0.34	0.75
Urine N:milk N	1.18	0.91	1.26	1.25	0.98	0.96	0.73	1.05	0.162	0.12	0.95	0.63
Manure N:milk N	2.3	2.0	2.5	2.4	1.9	2.1	1.8	2.2	0.22	0.21	0.59	0.51
MNE ¹	26.1	29.1	26.3	25.6	31.9	28.2	31.7	27.5	2.34	0.13	0.32	0.57

¹MNE = milk nitrogen efficiency (milk N:feed N).

decoupling urea formation from ruminal NH₃-N concentration (Poos et al., 1979).

Additionally, it is possible that Rumensin was systemically functional in a manner that retained urea-

N in the blood, potentially by acting on the kidneys or aquaporins throughout the body (Mollajew et al., 2010). However, no consistent effects of Rumensin on urea kinetics were observed in this study, indicating

Figure 1. Relationship between plasma urea-N (PUN) and rumen ammonia (NH₃-N) for animals consuming diets with (●) or without (■) Rumensin (Elanco Animal Health, Greenfield, IN). The equation describing the relationship in cattle fed Rumensin is $y = 0.658x + 6.3574$, $R^2 = 0.34$; the equation describing the relationship between PUN (mg/dL) and rumen NH₃-N (mg/dL) without Rumensin is $y = 0.7833x + 2.9214$, $r = 0.77$. Color version available in the online PDF.

that this either was not a possibility or that certain regulatory factors counteracted each other to result in similar urea fluxes among treatments.

Nitrogen Excretion

Among all diets, N intake was 129 g of N/d higher for the high CP diets, of which 59% was excreted in the manure (40% via urine and 19% via feces) and 11% in milk. Because these are the treatment mean averages for high versus low protein, they are relative in scale rather than descriptive of total N balance. The means are comparable to a series of trials feeding cows 8 to 12% MP (as % of DM), in which 83% of the increased MP was excreted in manure (61% via urine and 22% via feces) and 17% in milk (Weiss et al., 2009b). Feeding the high-protein diets resulted in an average of 82 g of N/d more manure N excretion (Table 6), primarily in the urine. Consistent with previous studies, excess N intake beyond that required for milk and growth was excreted in the manure and primarily through increased urinary N excretion, and differences in urinary N excretion were greater than those for fecal N excretion (Weiss et al., 2009b). Fecal N excretion was not significantly affected by dietary starch (Table 6).

Increases in urinary N excretion and PUN concentration with Rumensin feeding have been observed in previous studies (Poos et al., 1979; Martineau et al., 2007). Because Rumensin generally increased PUN concentrations in this study, we would expect to see correspondingly higher urinary N excretion; however, we found an inconsistent relationship between the 2 measurements, with Rumensin increasing urinary N excretion on the high-starch diets but having a negligible effect in low-starch diets (Table 6, interaction of starch and Rumensin, $P = 0.04$). This effect may be related in part to PUN concentrations but might also be a function of fecal and urine urea-N excretion, as increases in PUN did not consistently result in increased urinary urea excretion. What is clear from these results is that Rumensin facilitates a shift of ammonia out of the rumen and into the plasma, directly or indirectly, leading to higher observed PUN but not positively affecting urinary N excretion. Rumensin might also be affecting the animal systemically, but these results should be replicated with more animals in a focused study. Increases in fecal N with Rumensin also occurred in the high-protein diets, which might be a function of higher N intakes in those diets rather than a shift in N flows to the hindgut versus the rumen. Although Rumensin diets had numerically higher N excretion, approximately 21 g of N/d among all treatments, it also increased milk yield by 1.8 kg/d, resulting in no significant change in either urine N:milk N or manure N:milk N ratios (Table

6). The Rumensin-supplemented diets had numerically lower urine N:milk N ratios in the high-protein diets and relatively unchanged ratios in the low-protein diets.

Urea Kinetics

Urea-N entry rate (**UER**) was 25% higher ($P = 0.02$) and gastrointestinal tract (**GIT**) urea entry rate (**GER**) tended to be higher ($P = 0.07$) in cattle fed the high-protein diets (Table 7). Urea entry rate averaged approximately 57% of total N intake for cattle with and without Rumensin, and GER was similar at 43 and 42% of N intake for cattle fed and not fed Rumensin, respectively. These values were very similar to the average values described by Lapierre and Lobley (2001), where 58% of N intake was converted to urea and approximately 38% of N intake recycled to the GIT.

Various studies have demonstrated both urea synthesis and urea-N recycling to correspond to dietary N intake (Valkeners et al., 2007; Martineau et al., 2011). In this study, N intake was correlated with UER ($r = 0.81$) and GER ($r = 0.78$) and therefore the relationship between N intake and urea entry and partition was consistent with previous observations. Neither dietary starch nor Rumensin significantly affected any urea-N kinetic measurements (Table 7). This was unanticipated, because Rumensin-fed cattle were observed to have lower ruminal $\text{NH}_3\text{-N}$ concentrations, which should have resulted in lower UER and increased ruminal urea-N entry along with greater PUN concentrations, which would presumably stimulate urea-N excretion and increase ruminal urea-N entry (Sunny et al., 2007). Lower ruminal $\text{NH}_3\text{-N}$ concentrations have been observed to increase urea-N entry to the rumen (Marini and Van Amburgh, 2003), but it is also likely that the concentration gradient responsible for such N movement is most influential in the rumen epithelium rather than in the rumen fluid per se (Kristensen et al., 2010). Plasma urea concentration has also been observed to affect urea-N entry, as urea infusions lead to higher GER values (Sunny et al., 2007; Kristensen et al., 2010). It was our hypothesis that the ability of Rumensin to reduce rumen ammonia concentration would stimulate urea-N entry into the rumen or GIT, or both. However, this did not occur in this study, as none of Rumensin's effects on urea-N kinetics approached significance.

Based on the hypothesis and experimental design, a stimulatory effect of dietary starch on GER was anticipated, as previous studies have demonstrated increases in GER resulting from higher NFC fermentation (Kennedy, 1980; Gozho et al., 2008). Both UER and GER were numerically increased by high dietary starch in the high-protein diets, but were numerically decreased in the low-protein diets. Rapid starch fermentation or

Table 7. Urea-nitrogen recycling kinetics for lactating dairy cows supplemented with high or low dietary CP levels with or without Rumensin (Rum; Elanco Animal Health, Greenfield, IN) and high or low starch concentrations

Item ¹	High protein				Low protein				SEM	<i>P</i> -value		
	Rumensin		No Rumensin		Rumensin		No Rumensin					
	High starch	Low starch	High starch	Low starch	High starch	Low starch	High starch	Low starch				
No. of cows	3	3	3	3	3	3	3	2				
N intake, g/d	736.0	691.0	644.0	667.0	556.0	542.0	545.0	562.0	43	<0.001	0.84	0.14
UER, g of N/d	432.6	393.1	437.6	388.6	290.0	328.9	275.3	342.2	55.2	0.02	0.87	0.99
UER to urine (<i>u</i>)	0.293	0.252	0.254	0.291	0.335	0.243	0.247	0.242	0.049	0.88	0.40	0.55
UER to GIT (1 − <i>u</i>)	0.707	0.748	0.746	0.709	0.665	0.757	0.753	0.758	0.049	0.88	0.40	0.55
UUE, g of N/d	124.0	97.1	111.8	114.1	87.1	83.0	71.9	72.6	12.8	<0.01	0.20	0.22
GER, g of N/d	305.5	299.2	325.8	274.6	199.7	249.0	208.2	264.9	52.3	0.07	0.68	0.89
GER to ROC (<i>r</i>)	0.400	0.414	0.338	0.436	0.446	0.384	0.414	0.439	0.034	0.44	0.17	0.90
GER to UFE (<i>f</i>)	0.004	0.003	0.003	0.004	0.004	0.003	0.003	0.003	0.001	0.59	0.29	0.70
GER to UUA (<i>a</i>)	0.597	0.584	0.659	0.561	0.550	0.613	0.584	0.555	0.035	0.45	0.20	0.91
ROC, g of N/d	121.8	124.2	111.1	118.6	85.4	93.8	84.7	116.8	21.2	0.12	0.36	0.92
UFE, g of N/d	1.308	0.818	0.947	1.007	0.685	0.655	0.578	0.704	0.125	<0.01	0.29	0.55
UUA, g of N/d	182.4	174.1	213.8	155.0	113.6	154.6	122.9	115.4	30.3	0.04	0.65	0.84

¹UER = urea-N entry rate; UER to urine (*u*) = the proportion of UER that enters the urine; UER to gastrointestinal tract (GIT; $1 - u$) = the proportion of UER that enters the GIT; UUE = urinary urea elimination rate; GER = GIT urea entry rate, or recycled urea-N; GER to ROC (*r*) = the proportion of GER that re-enters the ornithine cycle; GER to UFE (*f*) = the proportion of GER that exits the animal in the feces; GER to UUA (*a*) = the proportion of GER that is utilized for anabolism; ROC = urea returning to the ornithine cycle; UFE = urea-N excreted in the feces; and UUA = urea-N utilized for anabolic purposes.

poor microbial capture of fermented energy might have led to the higher ruminal $\text{NH}_3\text{-N}$ concentrations and UER measurements observed with high dietary starch in the high-protein diets. However, the high-starch treatment did not affect the distribution of urea, as the partitioning of UER to the urine (*u*) or GIT ($1 - u$) were not affected, which might indicate that the starch was not fermented sufficiently in the rumen to stimulate urea entry into the rumen. It was expected that dietary starch, especially in the form of steam-flaked corn, would stimulate urea-N entry to the rumen via its effects on urea permeability of the rumen epithelium. Both VFA production and an increase in N gradient due to microbial capture of $\text{NH}_3\text{-N}$ would be expected to increase ruminal urea-N entry (Kennedy et al., 1981; Abdoun et al., 2006). Cattle fed the high-starch diets did demonstrate a trend to increase the use of recycled urea-N for anabolism instead of returning to the ornithine cycle for all diets except low-protein diets with Rumensin (Table 7). This suggests that dietary starch was able to stimulate microbial capture of some urea-N re-entering the rumen or potentially the hindgut if fermented postruminally. However, the effect of dietary starch on the total amount of urea-N for anabolism was not significant.

We observed a significant dietary starch and Rumensin interaction ($P = 0.01$), which resulted in Rumensin lowering the proportion of GER utilized for anabolism (*a*) in cattle fed high-starch diets but increasing it on low-starch diets (Table 7). Rumensin

also increased the proportion of GER exiting in the feces in cattle fed high-starch diets, but decreased it in those fed low-starch diets (Rumensin and starch interaction, $P = 0.01$). Corresponding changes were observed in the proportion of GER returning to the ornithine cycle (*r*), as $r + a + f = 1$, where *f* is the proportion of GER that exits the animal in the feces. Our hypothesis was that Rumensin would improve anabolic utilization of recycled urea-N by reducing ruminal deamination of feed protein and providing more opportunity for urea re-entering the rumen to be converted to microbial protein. Even though Rumensin decreased ruminal $\text{NH}_3\text{-N}$ concentrations, this did not consistently translate into more utilization of urea-N as would be expected from the competitive standpoint of similar amounts of GER with lower ruminal $\text{NH}_3\text{-N}$ concentrations. Rumensin might have affected the proportion of urea entering the hindgut, either by affecting urea transport systemically or simply through its effects on PUN concentration. The cattle fed the high-starch diets with Rumensin had increased PUN concentrations and might also have had proportionally higher hindgut urea entry and therefore lower anabolic utilization of urea. The proportion of GER that exited in the feces did increase when Rumensin was present in the high-starch diets (Rumensin and starch interaction, $P = 0.01$). As neither PUN nor ruminal $\text{NH}_3\text{-N}$ concentrations changed in parallel to these shifts in GER to the feces or for anabolism, it is difficult to determine the cause of these shifts.

Table 8. Correlation coefficients between various urea-N kinetic measurements in dairy cows by either level or dietary starch, Rumensin (Elanco Animal Health, Greenfield, IN), or among all treatments

Parameter ¹	Correlation coefficient				
	High starch	Low starch	No Rumensin	Rumensin	All treatments
UER: <i>u</i>	-0.27	-0.52	-0.18	-0.65*	-0.37*
UER:GER	0.98**	0.94**	0.96**	0.97**	0.97**
GER: <i>a</i>	0.62*	0.18	0.42	0.43	0.43*
GER:UUA	0.99**	0.93**	0.97**	0.97**	0.97**
GER: <i>r</i>	-0.62*	-0.18	-0.43	-0.42	-0.43*
GER:ROC	0.95**	0.84**	0.87**	0.90**	0.90**

¹UER = urea-N entry rate; *u* = the proportion of UER that enters the gastrointestinal tract (GIT); GER = GIT urea entry rate, or recycled urea-N; *a* = the proportion of GER that is utilized for anabolism; UUA = urea-N utilized for anabolic purposes; *r* = the proportion of GER that re-enters the ornithine cycle; and ROC = urea returning to the ornithine cycle.

* $P < 0.05$; ** $P < 0.001$.

The amount of urea-N utilized for anabolism was significantly higher for cattle fed the high-protein diets ($P = 0.04$). Although the partitioning of urea-N entering the GIT was not affected by dietary CP ($P = 0.44$ to 0.59), the much larger amounts of N entering the urea cycle and subsequent release in the GIT led to higher values overall (Table 8). These results suggest that dietary changes might in fact stimulate productive utilization of urea but these shifts in urea kinetics may be overshadowed by larger changes in dietary CP.

Gastrointestinal entry rate was highly correlated to UER among all treatments ($r = 0.97$; Table 8), indicating that dietary treatments did not have a significant effect on N recycling beyond their effects on urea synthesis. It has been hypothesized that urea-N moves across the rumen epithelium proportionally to the NH_3 gradient (Houpt and Houpt, 1968). Previous work has demonstrated an inverse relationship between the amount of urea-N entering the rumen and ruminal NH_3 -N concentrations, suggesting that urea flow is proportional to the N gradient (Kennedy, 1980). In this study, recycled urea-N (GER) and ruminal NH_3 -N concentrations were weakly correlated ($r = 0.40$), indicating that higher ruminal NH_3 -N concentrations corresponded to higher urea-N entry rates (Table 8). This suggests that N intake and total N pool sizes in the rumen and blood might overwhelm the more subtle mechanisms at the GIT epithelial border. This conclusion was supported by higher correlations between GER and PUN concentration ($r = 0.66$). Other work has also indicated PUN concentration in the short-term or N intake over the long-term, or both, to be more important determinants of urea entry rate than rumen NH_3 -N concentrations per se (Kristensen et al., 2010; Røjen and Kristensen, 2012). Therefore, PUN appears to be a better predictor of GER than ruminal NH_3 -N concentrations, although it is possible that both may have subtle effects on N entry (Marini and Van

Amburgh, 2003). Higher observed correlations might be due in part to the stability of PUN measurements compared with the measurement of ruminal NH_3 -N. An interesting observation in this study was that Rumensin appeared to shift N from ruminal NH_3 -N to PUN, but this was without concomitant increases in GER. Therefore, these data suggest altered transport of urea within the animal, but an explanation of the effect of Rumensin on urea transporters or kidney function is lacking in the literature.

Furthermore, rumen pH has been suggested to play a role in urea recycling via its effects on $\text{NH}_3/\text{NH}_4^+$ equilibrium and rumen wall solubility (Houpt and Houpt, 1968). In this study, pH was not correlated with GER among treatments ($R^2 = 0.06$; data not shown). Thus, within the conditions of this experiment, pH did not affect urea-N entry at a magnitude large enough to be observed. However, we did not observe a wide range of pH values and the lack of relationship is not surprising. More recent work has suggested that rumen fluid pH might not be as significant of a factor in $\text{NH}_3/\text{NH}_4^+$ transport across the rumen epithelium as its effect on local pH of the apical epithelial wall. This pH appears to be highly affected by VFA and CO_2 concentrations, and thus N transport is directly affected by $\text{NH}_3/\text{NH}_4^+$ concentrations and by these fermentation by-products (Abdoun et al., 2010).

The total amount of urea synthesized was expected to play a role in affecting its partitioning into the GIT or urine, with larger amounts resulting in less recycling and more excretion via the urine. This was true to some extent in this study, as the proportion of UER that entered the GIT (*u*) was moderately affected by the amount of UER ($r = -0.37$ among treatments; Table 8), with more urea synthesis resulting in a smaller percentage of urea entering the GIT. This relationship was stronger in diets with Rumensin ($r = -0.65$) and low starch ($r = -0.52$; Table 8). However, because of

Figure 2. The relationship between urea-N entry rate and gastrointestinal urea-N entry rate for each experimental unit across all dietary treatments differing in dietary CP, starch, and Rumensin (Elanco Animal Health, Greenfield, IN) inclusion fed to dairy cattle and continuously infused with $^{15}\text{N}^{15}\text{N}$ urea-N. In this experiment, gastrointestinal entry rate = $0.7319x - 21.27$, where x = urea entry rate; $r = 0.96$. Color version available in the online PDF.

the relatively small variation in treatment means for u and the lack of treatment effects, the amount of GER was correlated more closely to UER rather than to u , suggesting that urea-N recycling to the GIT can be reliably estimated by predicted urea synthesis, with only minor adjustments made by dietary factors. The data in Figure 2 demonstrate the consistency of this relationship by plotting UER and GER by individual experimental unit (each cow appears twice, once for each period).

CONCLUSIONS

Consistent with previous studies, supplementation with Rumensin resulted in a reduction in rumen NH_3 concentration but this reduction did not result in changes in urea production or urea N recycling and gastrointestinal entry rate. Lactating dairy cattle converted more than 50% of their N intake into urea and recycled approximately 73% of urea synthesized back to the GIT. This appears to be an obligate function to maintain NH_3 supply for gastrointestinal bacterial function. Rumensin appeared to have some physiological function beyond the ruminal effect on bacteria because the plasma N pool was larger without a concomitant shift in either urea-N recycling or urinary excretion.

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