Influence of diets supplemented with different nitrogen sources on rumen microbial protein synthesis and protozoa numbers in steers

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Abstract

An experiment with ruminally and duodenally cannulated steers was undertaken to determine how type of diets affected the ability of rumen fermentation to respond to different sources of nitrogen in the diet. Four steers received diets of corn or hay grass supplemented with either urea or casein in a 4 x 4 Latin Square Design with a 2 x 2 factorial arrangement. Measurements of protozoa numbers, rumen fermentation and microbial protein synthesis were made on the third week of each 21-day period. Ruminal pH was higher ($P<0.01$) for steers fed hay than corn diet. Concentrations of ruminal NH_3-N were higher for steers supplemented with urea than for steers supplemented with casein. Steers receiving corn or hay diets supplemented with casein had higher ruminal amino acid-N and peptide-N concentrations than those supplemented with urea. Nevertheless, steers receiving hay had 33% greater ($P<0.01$) MOEFF than steers receiving corn (18.61 vs. 14.03 g of microbial N/kg of OM digested). Total N flow to the duodenum was similar among treatments but non-ammonia non-microbial N flow to duodenum was higher ($P<0.0002$) in corn-based diet than hay-based diet. Despite having a lower ($P<0.01$) ruminal pH (6.21 vs. 6.52), steers fed the corn-based diet had greater ruminal organic matter digestibility than steers fed the hay-based diets, presumably reflecting a difference in the source of fiber in the two diets. Ruminal concentrations of total protozoa were higher ($P<0.01$) for corn-fed steers than hay-fed steers but holotrichs were more prominent in hay-fed steers. The source of N or peptides had no influence on protozoa numbers and MOEFF in the rumen. No benefit was noted in terms of ruminal or total tract digestion of organic matter or starch when a dietary source of peptides (casein) replaced dietary urea. Protozoa population in greater in corn based diets but their role in reducing rumen microbial synthesis is unclear in this experiment. Nevertheless, microbial efficiency was 32% greater with a hay-based than a corn-based diet.

Introduction

Ruminant animals derive their protein from undegradable dietary protein, microbial protein synthesized in the rumen, and endogenous protein. Under most dietary conditions, microbial protein constitutes a major source of protein (Ørskov, 1992;Posada et al., 2005). Great differences can exist in various feed protein in the contents of ammonia-N, peptides, free AA, amides, nucleotides and other nitrogenous compounds (Givens and Rulquin, 2004; Yan and Agnew, 2004), as well as the degradability of feed protein. Dietary structure also affects rumen fermentation and then alters the composition of rumen microbes (Brown et. al., 2006). This in turn affects the rumen environment and microbial population which might affect microbial protein flow to the duodenum. These changes will directly affect microbial protein synthesis and rumen N metabolism. Thus, there is some question about the value of different nitrogen sources in non-structural and structural carbohydrate based diets.

Ammonia is often the main nitrogen precursor for rumen microbial protein

synthesis under practical dietary conditions (Nolan, 1975; Aharoni et al., 1991). Growth of pure cultures of rumen bacteria is stimulated by peptides and amino acids (Chen et al., 1987; Cruz Soto et al., 1994). Many experiments showed that preformed amino acids and peptides increased the rate or efficiency of microbial growth compared to ammonia alone (Rooke and Armstrong, 1989; Merry et al., 1990; McAllan, 1991). However, not all studies have found that preformed amino acids and peptides were beneficial to rumen bacteria (Rooke and Armstrong, 1989; Cruz Soto et al., 1994). Cruz Soto et al., (1994) found that cellulolytic rumen bacteria actually had a variable response to amino acids and peptides: cellulose breakdown was unaffected, but their growth rate on soluble sugars increased several folds when preformed amino acids were present. They proposed that in general, rumen fermentation would be stimulated by preformed amino acids and peptides only when rapidly degraded energy sources are available (Cruz Soto et al. 1994).

Unlike bacteria, protozoa do not have urease (Onodera et al., 1988) and cannot use urea or ammonia to synthesize amino acids. Rumen protozoa obtained their protein needs by engulfing insoluble proteins in the rumen fluids. However, Newbold et al., (2005) recently derived evidence to suggest that protozoa can directly incorporate ammonia via glutamate dehydrogenase. Because NH_3-N assimilation into protozoal N has been assumed to be through bacterial (Koenig et al., 2000), the functional significance of this finding currently is not known. Clearly, the rumen protozoa-bacteria interactions need much more attention in the future to correlate rumen microbial numbers (and perhaps biomass) of protozoa and bacteria with microbial degradation of feed protein and microbial protein synthesis in the rumen. The objective of this experiment was to determine the effect of diets supplemented with different nitrogen sources on efficiency of microbial protein synthesis and protozoa numbers in the rumen of steers fed concentrate or roughage diets.

Materials and Methods

Animals and treatments

Four Simmental crossbred steers averaging 450 kg in weight were used. Each was equipped with rumen fistula and duodenal re-entrant cannula in the duodenum proximal to the bile duct. The steers were randomly allotted to individual 3 x 5 meter stalls and had free access to water. The steers were assigned to four treatments in a 4 x 4 Latin Square Design with 2 x 2 factorial treatment arrangement. Treatments included corn based diet + casein (CK), corn based diet + urea (CU), hay based diet + casein (HK) and hay based diet + urea (HU).

The ingredients and chemical composition of each diet are shown in Table 1.

Diets were fed twice daily at 0800 and 1600 in equal portions. Feed dry matter was provided at a rate of 1.8% of body weight daily. Chromium oxide (CrO) was used as a nonabsorbable marker for measurement of digesta flow. Chromium oxide (0.2% of the total diet) was mixed with the supplements for all diets.

Sampling procedures

Before the start of the experiment, the steers were fed with diet CU for a period of three weeks for diet adaptation. Each experimental period lasted 21 d long, with 16 d for adjustment and 5 d for sampling. On day 17 through 19, approximately 250 ml of duodenal digesta and 200 g of wet feces were collected at 2 and 8 h after feeding. On day 20, approximately 1000 ml of strained rumen fluid were collected at 2 and 8 h after feeding and frozen for later isolation of bacteria. On day 21 approximately 250 ml of rumen fluid were withdrawn at 1, 2, 4 and 8 h after feeding. A 0.5 ml sub-sample of the rumen fluid from each sampling time was added to a formaldehyde/phosphate buffer/methyl green solution according to a procedure developed by Department of Animal Sciences and Industry, Kansas State University and stored later for protozoa counting. The remaining samples were frozen and were

later analysed for ammonia and peptides. All rumen fluid collected was strained through 4 layers of cheese cloth and the pH was measured immediately. Before freezing, all rumen samples were acidified with 1 ml of 20% v/v sulfuric acid per 50 ml strained fluid to stop microbial activity.

Feed samples were obtained prior to each sampling day and composited within each diet and period. All samples were ground in Wiley Mill fitted with a 2 mm screen and stored for analysis.

Laboratory analyses and calculations

Feed, duodenal and fecal samples were analyzed for dry matter (DM), organic matter (OM), ash (AOAC, 1984), starch (Herrera-Saldana and Huber, 1989) and chromium (Cr) (Fenton and Fenton, 1979). The N content of feed, duodenal digesta, bacterial composites, and feces were analyzed by macro-Kjeldahl analysis (AOAC, 1984). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) in feed, duodenal and fecal samples were analyzed using procedures of Goering and Van Soest (1970). Rumen NH_3-N was analyzed colorimetrically using a spectrophotometer (UV-VIS Spectrophotometer, Gilford-Respond Series 1987) following the procedures of Broderick and Kang (1980). Protozoal numbers in the rumen fluid were counted using a Sedgewick Rafter chamber. Bacteria were isolated from the ruminal fluid using the procedures of Weakley and Owens (1983). Dried duodenal and bacterial samples were analyzed for nucleic acid-N by the procedure of Zinn and Owens (1986). To improve recovery of RNA pellets after precipitation with silver chloride, the RNA pellets were washed with a solution (100 ml) that consisted of 5 ml solution containing 12.5% $HCLO_4$ in .0285M $NH_4H_2PO_4$ + 5 ml of 0.4 M $AgNO_3$ + 90 ml of 0.2 M $NH_4H_2PO_4$. Rumen samples for peptide-N analysis were prepared using the procedures of Chen et al., (1987). Prehydrolyzed and hydrolyzed rumen fluid samples were analyzed colorimetrically at 570 nm using a spectrophotometer (UV-VIS Spectrophotometer, Gilford-Respond Series 1987). The concentrations of ninhydrin reactive material in hydrolyzed and unhydrolyzed were measured and leucine was used as a standard (Moore and Stein, 1954; Moore, 1968). The concentration of peptide-associated α-amino N was calculated as the difference between the α-amino N content of hydrolyzed and the unhydrolyzed samples. The concentrations of α-amino N in the hydrolyzed and unhydrolyzed were corrected for NH_3-N in samples and ninhydrin (by subtracting NH_3-N concentrations in the samples and ninhydrin from α-amino N concentrations in the hydrolysed and unhydrolysed samples).

Flows of DM at the duodenum were calculated by dividing daily Cr intake (g) by Cr concentration (g/kg) in duodenal digesta. Nutrient flows were calculated by multiplying DM flow by the concentration of the given nutrient in duodenal DM. Daily N flow at the duodenum was partitioned into microbial N, ammonia N and non-ammonia non-microbial N (NANMN). Bacterial DM was determined by oven drying the freeze-dried bacteria samples (ground) at 60 °C for 24 h. Daily duodenal organic matter (OM) flow, corrected for microbial contribution, was calculated from the corrected duodenal DM flow multiplied with duodenal OM percentage. Efficiency of microbial protein synthesis (MOEFF) was expressed as gram of N per kilogram of OM truly digested in the rumen. Microbial N was determined by multiplying the purine concentration in the duodenum by the purine to N ratio in the ruminal bacterial pellet (Zinn and Owens, 1986) isolated from each steer in each period. Ammonia N flow was determined by multiplying the ammonia N concentration times the liquid flow rate. Flow of NANMN was determined by subtraction of the microbial and ammonia N from total N. Microbial efficiency was calculated by dividing microbial N flow by OM truly fermented in the rumen. Apparent total tract N digestibility (ATTND) was calculated as intake of N minus fecal N divided by intake of N.

Statistical analysis

Variables measured were analyzed as 4 x 4 Latin square with animal (4), period (4) and dietary treatment (4) as factors (SAS Institute, Inc. 1985). Differences between treatments were determined using a multiple comparison test (PDIFF options of SAS Institute, Inc. 1985). Statistical significance was considered to exist where $P<0.05$, whereas a trend was considered to exist if $0.05 \leq P \leq 0.10$. Simple correlations were calculated across all observations.

Results and Discussion

Mean ruminal pH, peptide-N, amino acid-N, and NH_3-N concentrations are as shown in Table 2. Ruminal pH was higher ($P<0.01$) for steers fed hay than corn diet because the hay diet contained a large amount of fiber that stimulated rumination and increased saliva flow to the rumen. Concentrations of rumen NH_3-N were higher ($P<0.05$) for steers supplemented with urea than for steers supplemented with casein. Mean concentrations of ruminal NH_3-N for treatments CK, CU, HK and HU were higher than the concentration adequate (5 mg/dL) for supporting microbial crude protein synthesis (Satter and Slyter, 1974). These values were within the values (3.3 to 8.5 mg/dL) suggested by Kang-Merznarich and Broderick (1981) but the values were lower than the range (6-30 mg/100 ml) reported by Devant et al. (2000) for maximal microbial growth when diets contained 74% corn grain. Ammonia level was maximal at 2 h in steers supplemented with casein and remained slightly lower at 4 and 8 h. After 2 h of feeding CU and HU diets, ruminal NH_3-N was very high then rapidly declined thereafter indicating less N was available to rumen microorganisms. The more rapid accumulation of NH_3-N with urea than with casein reflected slight resistance of casein to ruminal degradation (Wallace et al., 1987). Ruminal amino acid-N concentration was higher ($P<0.01$) for steers supplemented with casein when fed either corn or hay diets (Table 2). The values of amino acid-N in all treatments were low confirming the results of Ives et al. (2002) who fed cattle with widely different diets. Amino acid-N peaked rapidly after 1 h of feeding in steers supplemented with casein before declining rapidly thereafter suggesting that amino acid-N always remained available to microorganisms as N source but the level was always low 1 h post-feeding. Steers receiving hays supplemented with urea had slightly higher ($P<0.10$) peptide-N concentration than steers receiving corn supplemented with urea because hay in general has higher ruminally degraded protein than corn.

MOEFF were 14.2, 13.9, 18.8 and 18.4 for steers fed diets of CK, CU, HK and HU, respectively (Table 2). Those steers receiving hay diets supplemented with either casein or urea had higher ($P<0.01$) MOEFF than steers receiving corn supplemented either casein or urea. The source of N did not ($P<0.62$) affect MOEFF and there was no energy-protein interaction among treatments. Lower MOEFF with the corn based diet than hay based diet indicated that diets which contained more than 70% ground corn might cause rapid rate of nonstructural carbohydrate degradation in the rumen, resulting in uncoupled fermentation (Polan, 1988). Uncoupled fermentation was presumed to occur when energy was released much faster than it could be captured and utilized by the ruminal bacteria (Clark et al., 1992). For the hay based diet, one would expect greater saliva flow, higher ruminal pH, an improved cation exchange capacity, improved hydration, and an improved ruminal mat formation. This would decrease liquid retention times that would increase microbial growth as microbial generation times would be reduced (Sniffen et al., 1992). The effect of readily fermentable carbohydrate supplementation on the efficiency of microbial protein synthesis might depend on the level of supplementation. Efficiency increased when readily fermentable carbohydrate was supplemented with less than 30% of the total diet (Feng et al., 1993), but decreased when the

supplementation level was greater than 70% (Mathers and Miller, 1981). In contrast, Sahoo and Walli (2008) reported higher microbial protein yield from kids given different rumen degradable protein as compared to kids receiving rumen undegradable protein in high energy concentrate diets. The correlation between peptide-N concentrations and MOEFF was low (r = 0.07, P= 0.8). Although peptide-N concentration was highest in steers supplemented with casein, it did not appear to stimulate microbial growth in this experiment. Cruz Soto et al., (1994) also presented evidence that stimulation by peptides and amino acids would not always occur. Supplying peptides with diets containing either rapidly or slowly degraded fibers showed that the benefits of peptides would be evident only when the energy source supported a growth rate which enabled the organism to respond (Chikunya et al., 1996). Bach et. al. (2005) reported that low concentration of peptides and amino acids could potentially limit microbial growth when fed diets rich in non-structural carbohydrates. Similarly, Atasoglu et al. (2004) reported that some amino acids limited the growth of ruminal bacteria. Microbial growth was improved when peptides or amino acids replaced urea or ammonia as sole or major source of N (Maeng et. al., 1989). However, Ipharraguerre et. al. (2005) studied the effects of ruminal fermentation and intestinal supply of nutrients in dairy cattle suggested that the degradability of dietary protein which affected the availability of ruminal peptide-N might modulate the microbial protein outflow to the duodenum. In vitro experiment by Li (2001) revealed that bacterial protein yields of soybean meal and fish meal (high degradable protein) were higher than that of feather meal (low degradable protein). In the present trial, steers receiving hay appeared to have an energy source that supported greater microbial growth efficiency regardless whether there was high rumen peptide-N concentrations or not. Although the rumen NH_3-N and peptide-N concentrations were high in corn fed steers, the lower bacterial yield and efficiency with this diet indicated that factors other than ruminally available energy, ammonia and peptides limited microbial growth and efficiency.

Total N flow to the duodenum was affected by the source of energy (Table 3). Nitrogen flow to the duodenum was greater (P<0.0001) for steers fed corn than for steers fed with hay diet. Compared with urea, casein supplementation also increased (P<0.02) N flow to the duodenum. The source of energy affected (P< 0.04) microbial N flow to the duodenum. Hay diet fed steers had higher flow than steers fed corn diet but the effect of N source was not significant (P<0.12) suggesting that nitrogen source or peptides had no effect on microbial N flow. Microbial N accounted for 49.9, 47.0, 61.3 and 61.6% of total N flow to the duodenum for treatments CK, CU, HK and HU, respectively. NANMN flow to the duodenum was greater (P<0.0002) for steers fed corn diets than those steers fed hay diets but the effect of N was small (P<0.51). Steers fed the hay diet had higher (P<0.0001) ruminal N digestibility than corn fed steers (Table 3*).* The source of N had no effect on ruminal N digestibility (P<0.17) suggesting that ruminal peptides did not play an important role in ruminal N digestibility. ATTND was unaffected by N source but was influenced by energy source (P<0.0001). It was higher in steers receiving corn diets than those receiving hay diets. Post-ruminal supply of digestible N was about 50% higher in corn than hay diets (106 vs. 70 g/d). Protein-energy interactions for all these measurements were insignificant. Fecal N excretion were higher (P<0.05) in steers fed hay diet than corn diet and were not affected by N sources. These results were in agreement with those reported by Carro et al. (2000) and Moorby et al. (2006) in their studies on sheep fed different forage and concentrate ratio supplemented with different protein sources.

Basal diet affected the ruminal concentrations of the major protozoa species numbers (Table 4). The protozoa population increased after feeding and then became constant thereafter in all treatments. The

concentrations of entodiniomorph were higher (P<0.001) in the rumen of steers fed corn-based diets than in the rumen of steers fed with hay diets. An effect of N sources also was detected (P<0.02) as well as a protein-energy interaction in an entodiniomorph population. The population density of rumen protozoa in ruminants fed with forage plus concentrate was generally higher than that in ruminants fed with forage only (Nakamura and Kanegasaki, 1969). Dennis et al., (1983) fed Holstein heifers diets that varied in the ratio of roughage to concentrate: 70:30, 50:50, and 30:70 and found that the numbers of protozoa increased as proportion of concentrate increased in the diet. Meyer et al. (1986) reported that protozoa numbers increased with each increment in ground corn given to sheep. This undoubtedly is a reflection of dietary quality and the readily available highly digestible carbohydrate in corn based diets, which will increase entodiniomorph numbers. In contrast feeding 100% concentrate diets rich in fermentable carbohydrate decreased the protozoal population in sheep (Sutton et al., 1983). Total concentration of protozoa in ruminal fluid generally increased with the addition of concentrate to forage diets (Franzolin and Dehority, 1996; Brossard et al., 2003), but feeding high-grain diets resulted in reduced protozoa numbers or even defaunation (Hristov et al., 2001). Meng *et al*. (2000) reported that protozoal count was lower when total rumen degradable protein was supplied from urea in continuous culture fermenters.

Holotrich protozoa numbers were higher (P<0.0001) for hay-fed steers than for corn-fed steers with N source also playing highly significant role (P<0.007) although the total protozoal population was not significantly altered by N source (P < 0.13). The lower total protozoa numbers in steers fed with hay diet might be due to increase microbial flow but not greater with the hay diet, only MOEFF was. Steers (Smith et al., 1978) and sheep (Sutton et al., 1983) were also found to have increased MOEFF when the number of protozoa was reduced. According to Jouany et al. (1988), protozoa provided only about 20% of the total microbial N entering the duodenum of the ruminant, which did not reach the level expected given the protozoa/bacteria biomass ratio in the rumen. Protozoa can comprise half of the biomass (Michalowski, 1989). Ivan et al. (1992), evaluating the inoculation of fauna-free wethers with different ciliate genera, reported a decrease in the flow of bacterial N of nearly 45% after inoculation with cilates. The reduction in non-ammonia N flow was higher than 20% and the reduction in total AA flow reach 30% of the various genera, Holotrich protozoa showed the smallest impact on nitrogen metabolism.

Conclusion

Efficiency of converting digested organic matter to microbial protein in the rumen of steers was not increased by an increased supply of peptides with either hay or corn diets. Rumen microorganisms in corn-based diets may not require peptides as N source to synthesize their protein when an adequate supply of NH_3-N is available. Consequently, the degradable protein content in the concentrate should be considered before supplementation with highly degradable protein is exercised. No benefit was noted in terms of ruminal or total tract digestion of organic matter or starch when a dietary source of peptides (casein) replaced dietary urea. Protozoa population was greater in corn based diets but their role in reducing rumen microbial synthesis is unclear in this experiment. Nevertheless, microbial efficiency was 32% greater with a hay-based than a corn-based diet.

Table 1. Composition of diets fed to steers

Ingredients	Diets (% of dry matter)			
	Corn + Casein	Corn + Urea	Hay + Casein	Hay + Urea
Ground corn	74.79	77.99	_	_
Cottonseed hulls	11.0	11.0	_	_
Brome hay	_	_	25.0	25.0
Prairie hay	_	_	60.8	64
Supplements:				
Soybean hulls	7.0	7.0	7.0	7.0
Urea	_	1.4	_	1.4
Casein	4.6	_	4.6	_
Dicalcium phosphate	1.4	1.4	1.4	1.4
Limestone, 38%	0.7	0.7	0.7	0.7
Trace mineralized salt	0.3	0.3	0.3	0.3
Vitamin A	0.01	0.01	0.01	0.01
Chromic oxide	0.2	0.2	0.2	0.2
Crude protein, %	12.3	12.7	12.2	12.4
Starch, %	54.2	56.5	2.9	2.9

Table 2. Rumen pH, ammonia-N, peptide-N, amino acid-N and microbial protein synthesis in steers fed diets of grass hay or corn supplemented with either urea or casein

Item	Diets				SEM
	Corn + Casein	Corn + Urea	Hay + Casein	Hay + Urea	
pH	6.18[a]	6.24[a]	6.58[b]	6.47[b]	0.04
NH_3-N, mg/dL	7.55[a]	8.55[b]	7.40[a]	8.68[b]	0.23
Amino acid-N, mg/L[1]	3.89[a]	2.32[b]	3.95[a]	2.36[b]	0.13
Peptide-N, mg/L[2]	56.50[a]	1.99[b]	56.24[a]	4.21[b]	1.81
Microbial efficiency[3]	14.18[a]	13.88[b]	18.78[b]	18.38[b]	0.65

[ab] Means in the same row with different letters differ significantly ($P<0.05$)
[1] Prehydrolysed fluid
[2] Hydrolysed fluid
[3] g Microbial N per kg OM fermented

Table 3. Nitrogen digestion in steers receiving diets of grass hay or corn supplemented with either urea or casein

Item	Diets				SEM
	Corn + Casein	Corn + Urea	Hay + Casein	Hay + Urea	
Nitrogen intake, g/d	162.8	163.1	162.6	162.7	-
E. duodenum, g/d[1]	149.6[a]	144.3[a]	136.1[b]	131.6[b]	2.2
Microbial N, g/d	74.5[a]	67.8[a]	83.4[b]	81.1[ab]	2.4
NANMN, g/d[2]	66.9[a]	66.4[a]	42.4[b]	38.5[b]	2.7
Ruminal digestion, %					
Unadjusted	8.1[a]	11.5[b]	16.3[c]	19.1[d]	-
Adjusted[3]	58.9[a]	59.3[a]	73.9[b]	76.3[b]	-
Fecal N, g/d	39.5[a]	41.6[a]	63.2[b]	63.8[b]	2.2
ATTND, %[4]	75.8[a]	74.5[a]	61.1[b]	60.8[a]	1.4

[abcd]Means in the same row with different letters differ significantly ($p<0.05$)
[1] Entering duodenum
[2] Non-ammonia non-microbial nitrogen
[3] Adjusted for microbial and ammonia nitrogen
[4] Apparent total tract nitrogen digestibility

Table 4. Least square means of ruminal protozoa numbers in rumen fluid from steers receiving diets of grass hay or corn supplemented with either urea or casein

	Diets			
	Corn + Casein	Corn + Urea	Hay+ Casein	Hay + Urea
Protozoa				
Entodiniomorph	50.18[a]	54.02[b]	11.63[c]	12.21[c]
Holotrich	0.96[a]	0.55[b]	2.36[c]	1.45[d]
Total	51.14[a]	54.57[b]	13.99[c]	13.66[c]

[abcd]Means in the same row with different letters differ significantly ($p<0.05$)

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