Comparison of Nitrogen-15 and Diaminopimelic Acid for Estimating Bacterial Protein Synthesis of Lactating Cows Fed Diets of Varying Protein Degradability[1]

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ABSTRACT

Three lactating Holstein cows fitted with duodenal cannulae were fed diets containing cottonseed meal, corn gluten meal, or blood meal as protein supplements in a 3 × 3 Latin square experiment. Diets averaged 15% CP and were 60% concentrate, 31% corn silage, and 9% alfalfa hay. The flow marker was Cr_2O_3; the bacterial protein fraction of digesta CP was estimated by ^{15}N (as ammonium sulfate) and diaminopimelic acid. The undegraded fraction of total feed protein entering the duodenum for respective diets was .52, .57, and .69. The ^{15}N method was less variable than diaminopimelic acid. Based on ^{15}N, percentage of bacterial of total protein differed among treatments (61.5, 59.4, and 55.0, respectively). Ten percent more protein entered the duodenum on blood meal than other diets, but differences were not significant. Protein sources were similar in microbial passage, but degraded protein was used most efficiently for microbial synthesis on blood meal.

Incorporation of ^{15}N consumed into bacterial protein ranged from 50 to 83% with numerically highest values on blood meal, suggesting greater efficiency of ammonia capture. Recoveries of ^{15}N for the 72 h as milk, feces and urine ranged from 54 to 78%.

(**Key words**: bacterial protein, diaminopimelic acid, protein degradability)

INTRODUCTION

Estimates of duodenal flow of protein from dietary sources varying in degradability are limited (17) and often difficult to interpret because of contradictions associated with animals, diets, management methods, and the several microbial marker techniques (29). Diaminopimelic acid (DAP) is a common bacterial marker, but values obtained for proportion of microbial protein with DAP were higher (27), similar (23), and lower (16) than those based on ^{35}S. Other studies have confirmed lack of precision of various markers (27).

The objective of this study was to compare DAP and ^{15}N as bacterial protein markers for lactating cows receiving diets containing three protein sources of varying degradabilities. The ^{15}N method was preferred to other isotopic markers because of its stable nature, ease of handling, and minimal problems in disposition of wastes and tissues. Concentration of ^{15}N in various biological samples and recovery patterns for feces, urine, and milk were monitored to evaluate further this isotope as a bacterial marker when administered orally.

MATERIALS AND METHODS

Animals, Treatments, and Sampling

Three lactating Holstein cows, fitted with T-type cannulae in the proximal duodenum, were fed diets containing cottonseed meal (CSM), corn gluten meal (CGM), or blood meal (BM)

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in a 3 × 3 Latin square experiment. Completely mixed diets consisted of 60% concentrate, 31% corn silage, and 9% chopped alfalfa hay and were formulated to contain 15% CP (of DM) with 45% of the total CP from the different supplements. Table 1 gives ingredient composition of concentrate mixes and nutrient composition of complete diets. The BM diet had slightly higher CP and NDF but lower ether extract and ADF than other diets.

Cows were housed in separate stalls, had free access to water and were fed for ad libitum access (to allow 5% orts) twice daily at 0700 and 1700 h. Each experimental period consisted of 14 d of adjustment and 6 d of sampling. However, due to a health problem during one period, two cows were temporarily removed from the experiment and allowed to recuperate on a conventional diet of predominantly alfalfa hay, corn silage, and wheat straw. These cows were gradually returned to treatment diets before beginning the 14-d adjustment. The study spanned 15 wk from April to July 1986, but each cow completed her sequence of rations in 10 wk.

Chromic oxide was mixed with wheat flour (1:4) and water, baked at 100°C until dry, and pulverized with mortar and pestle. The Cr_2O_3 mixture was then incorporated at 25 g Cr_2O_3/d into the total diet starting 7 d prior to the collection. During sampling, ammonium sulfate (10% ^{15}N) was added to furnish 600 to 800 mg ^{15}N daily to all diets. The larger amount of ^{15}N was used early in the trial but was reduced as a conservation measure after initial analyses of digesta indicated that enrichment was sufficient to lower amount of isotope fed. The ^{15}N was administered by dissolving enriched ammonium sulfate in water to give about 200 ml of solution, which was sprayed onto 1 kg of minced corn silage. The corn silage was mixed daily into the complete diet.

TABLE 1. Composition of ingredients of concentrate mixes and of nutrients in complete diets.

Ingredients	Diet[1]		
	CSM	CGM	BM
	(% of DM)		
Protein supplement	24.8	18.3	14.5
Corn, ground; shelled	69.4	63.9	66.0
Urea	.5	.5	.5
Soybean hulls	. . .	7.6	7.8
Molasses	. . .	4.5	4.6
Minerals	5.2	5.3	6.6
Nutrients			
Crude protein	14.9	14.5	16.4
Ether extract	3.5	2.5	2.2
ADF	17.0	17.1	14.9
NDF	25.8	26.4	29.3
Lignin	3.9	3.4	2.2
Cellulose	10.8	10.4	8.8

[1]CSM = Cottonseed meal; CGM = corn gluten meal; BM = blood meal.

Every day, weights of feed offered were recorded and whole mixed diets were sampled. Orts were removed, weighed, and sampled before the morning feeding. Feed and ort samples were prepared for analysis by oven drying at 55°C and grinding in a Wiley Mill (Thomas-Wiley Laboratories, Swedesboro, NJ) fitted with a 2-mm screen. For Cr_2O_3 analyses, samples were ground through a cyclone grinder. Chemical analyses were on period composites.

Cows were milked twice daily and milk weights were recorded. Milk was sampled for composition analysis at each milking during digesta sampling. Prior to commencement of each sampling period, an indwelling urinary catheter was inserted into each cow. Urine was collected into 20-L plastic containers to which 10 ml concentrated H_2SO_4 had been added. A 1% aliquot by weight of daily urine output was retained for analyses. Fecal grab samples were taken twice daily, oven-dried at 55°C, and ground in a Wiley Mill through a 1-mm screen.

Duodenal digesta were sampled at intervals of approximately 4 h for 72 h. Because of unsteady flow patterns into cannulae, small fluctuations in time of sampling occurred. Digesta samples were stored in a refrigerator (5°C) for 4 to 48 h, but generally not more than 12 h, prior to isolation of bacteria. Two to three consecutive samplings were composited to yield eight subsamples per cow for each diet. These composites represented time spans of 7 to 12 h. About two-thirds of each subsample was mixed in a Waring blender at low speed for 20 s to dislodge bacteria associated with rumen particulate matter and then strained through four layers of cheesecloth. The strained fluid was centrifuged at 4000 × g to remove feed particles and protozoa. The supernatant was then centrifuged at 27,000 × g to collect bacteria. After washing twice with distilled wa-

ter, the bacterial pellet was oven-dried at 55°C. It was later ground to a fine powder with mortar and pestle.

The remaining digesta sample was dried at 55°C and ground to a fine powder in a cyclone grinder. Weighted portions of these digesta samples were pooled in proportion to number of hours represented to form one composite representing the entire period for each cow on each diet. For comparison of DAP estimates using ruminal and duodenal bacteria, rumen samples were drawn by stomach tube from each cow several times, except for one cow fitted with a rumen fistula, during each period for preparation of a composite bacterial pellet for each period using methods similar to those described for the duodenal bacterial pellets. For the purpose of studying ^{15}N decay patterns, milk, digesta, and feces were sampled for 120 h and were analyzed only for ^{15}N.

Analytical Methods

Feed, orts, digesta, and feces were dried at 100°C in a vacuum oven for DM determination, but other analyses were performed on samples dried at 55°C and expressed as fractions of DM determined at 100°C. A nitrogen autoanalyzer employing the Kjeldahl procedure was used for CP determinations. The Kjeldahl digestate was the initial step in processing samples for ^{15}N and Cr_2O_3 analyses. Analyses of ADF, lignin, and cellulose were according to Goering and Van Soest (9), NDF according to Robertson and Van Soest (22), and ether extract by AOAC (2). Composition of milk for fat, protein and lactose was determined at the Arizona DHIA laboratory, Phoenix, using infrared analysis.

For ^{15}N analysis, acid digesta from the Kjeldahl procedure was steam distilled and ammonia was collected in sulfuric acid. The ^{15}N content was determined by mass spectrometry as described by Pessarakli (19). For Cr_2O_3 analysis, 5 ml of deionized water and .4 to .5 g of periodic acid were mixed with the acid digesta from Kjeldahl and boiled for 4 h. Analysis of Cr_2O_3 was by atomic absorption spectrometry using a multielement hallow cathode lamp at 357.9 nm with an air:acetylene flame after digestion with sulfuric acid and redigestion with periodic acid.

For analysis of DAP, a 100-mg sample was hydrolyzed by heating overnight in a water bath in 25 ml of 6 N HCl. After hydrolysis, HCl was removed by vacuum evaporation, and 10 ml of sodium citrate buffer (pH 2.2) were added to the hydrolysate mixture, which was then filtered through a Whatman #5 filter paper (Whatman, Clifton, NJ). Amino acid separation was with a Beckman Model 121 automatic amino acid analyzer (Beckman Instruments, Fullerton, CA) with a two-column gradient buffer system and a Beckman W-1 ion exchange resin using the ninhydrin reaction for detection and quantification. Column temperatures were maintained at 55°C, and flow rates were 70 and 35 ml/h for the buffer and ninhydrin, respectively. The DAP eluted on the long column between valine and methionine at a pH of about 3.3.

Calculations

Dry matter flows were calculated using Cr_2O_3 as digesta marker. A steady state in marker passage during the sampling period was assumed. Marker concentration in ingested feed was adjusted for Cr_2O_3 of orts. Bacterial CP of duodenal digesta CP (BCP/DCP) was determined by two methods, ^{15}N and DAP. For both methods, values for bacterial samples were matched with digesta samples from which they were isolated.

For the ^{15}N method, the formula was:

$$^{15}N \text{ enrichment of digesta} \div {}^{15}N \text{ enrichment of bacteria.}$$

Enrichment was defined as the percentage of ^{15}N occurring in biological samplings in excess of that occurring in nonenriched ammonium sulfate, which averaged .36% of the total N, but blanks were subtracted from every sample analyzed for ^{15}N. Due to large errors in instrument sensitivity at low enrichment only digesta and bacterial samples taken 12 h and longer after initiation of feeding ^{15}N were used in calculating estimates of BCP/DCP. For the DAP method, the formula was:

$$\text{DAP-N:total N of digesta} \div \text{DAP-N:total N of bacteria.}$$

The individual estimates of BCP/DCP were averaged to obtain mean values for each cow on each diet. A weighted mean was obtained according to the number of hours represented

by the respective samples which was used in statistical analysis. For comparing variability between methods, unweighted means and associated coefficients of variation were used. Organic matter (OM) content of rumen bacteria was estimated according to (18).

Statistical Methods

Partitioning of variability was by ANOVA for a 3 × 3 Latin square according to Cochran and Cox (4). Duncan's multiple range test was used for separation of treatment means whenever the ANOVA showed significant treatment effects.

RESULTS AND DISCUSSION

Intake and Digestibility

Mean daily intakes, digestibilities for upper tract of DM and CP, and digestibilities for lower and total tract of CP are in Table 2. Treatment effects were in the direction expected from examination of literature values (18) but were not different ($P<.10$). For the BM diet, DM intake was slightly lower but CP intake was similar to other diets, due to a higher CP content. After correction for bacterial CP synthesis by the ^{15}N method, fractions of the total feed protein as undegraded protein entering the duodenum for the BM, CGM, and CSM diets were .52. .57, and .69, respectively. These values appear similar to literature estimates, considering that the three supplements fed provided 45% of the total N in the diets (18). The negative apparent digestibilities of CP indicate a large net gain of N in the forestomachs, apparently due to recycling (17).

Increase of N flow over N intake at the duodenum for the BM diet equalled 50% of dietary N intake, and values for CGM and CSM were about 30%. These results suggest greater recycling of N as rumen degradability of dietary CP decreased. Low rumen ammonia concentrations have been associated with slow degradation of protein (8, 11, 25, 26) and promote increased recycling of nitrogen (13, 14). However, rumen ammonia was not measured in this study, because passing of stomach tubes might have disrupted infusion.

Milk Production and Composition

Production and composition of milk (Table 3) showed no diet effect ($P>.10$), but there was a tendency for higher yields and lower milk fat and protein on CSM. Low cow numbers and high variation preclude conclusions on diet effects.

Microbial Protein Synthesis

Unweighted estimates of BCP/DCP for the different marker methods for each cow on each

TABLE 2. Effects of dietary protein supplement on intake and upper, lower, and total tract digestibilities.

Item	Ration[1]			SEM
	CSM	CGM	BM	
Intake, kg/d				
DM	16.78	15.90	14.38	1.57
CP	2.51	2.32	2.36	.94
Upper tract digestibility, %				
DM[2]	46.74	54.41	52.35	6.15
CP[3]	-34.49	-39.04	-54.18	8.88
CP[2]	48.38	43.29	31.20	4.08
Lower tract digestibility of CP,[3]%	64.55	64.34	57.52	5.33
Total tract digestibility of CP,[3]%	52.85	50.10	34.11	8.62

[1]CSM = Cottonseed meal; CGM = corn gluten meal; BM = blood meal.

[2]Calculated by correcting for bacterial fraction in duodenal digesta as estimated by the ^{15}N marker method.

[3]Apparent digestibility.

TABLE 3. Effect of dietary protein supplement on milk production and composition.

Item	Ration[1]			SEM
	CSM	CGM	BM	
Milk, kg/d	27.47	22.53	21.37	4.06
Fat %	3.38	3.79	3.86	.09
Protein %	2.76	2.98	2.92	.14
Lactose %	5.27	5.32	5.06	.07

[1]CSM = Cottonseed meal; CGM = corn gluten meal; BM = blood meal.

diet are in Table 4. Considerable variation existed within animals, which could have resulted from changes in the microbial protein flow at different sampling times. Variability was consistently lower for the ^{15}N method. This agrees with other reports suggesting that isotopic markers are less variable than internal diet markers for estimating BCP/DCP (16, 27). The relatively high variability associated with DAP might relate to its inherent limitations as a bacterial marker. The ratio of DAP N:total N of bacteria varies with bacterial species (7) and with diet (3, 7). Bacteria attached to plant fibers or the fluid phase might also contribute to the variability of this method (14, 15). Further, DAP was shown to be present not only in bacteria but in feedstuffs (7, 21) and protozoa (12, 16, 21). Hence, nonbacterial DAP in digesta might contribute to the variability of the estimate.

Using ruminal DAP concentrations to estimate duodenal BCP resulted in values about 15% higher with greater variability than duodenal DAP. Fewer samples and random sampling might have caused this discrepancy. Estimates of BCP/DCP from duodenal DAP analyses were much closer to values derived from ^{15}N procedures.

Table 5 shows mean DAP-N:total N ratios of duodenal digesta and bacteria. There were no animal or diet effects ($P>.10$). Both markers gave BCP/DCP values within a similar range but ranked diets in opposite directions. In contrast to DAP, the ^{15}N revealed differences ($P<.05$) between diets in the expected order based on reported degradabilities of the protein supplements compared (17). These data indicate limitations of DAP as a microbial marker.

Based on the ^{15}N method, BCP/DCP was lower ($P<.05$) for the BM than CSM diet, but differences were not significant between CSM

TABLE 4. Unweighted estimates for individual cows of percentage bacterial crude protein in crude protein of duodenal digesta (BCP/DCP) by two marker methods.[1]

Diet[2]	Period	Cow	Microbial marker					
			Nitrogen-15			Diaminopimelic acid		
			Mean (%)	SD	CV ((%)	Mean (%)	SD	CV (%)
CSM	1	932	57.7	6.9	11.9	55.2	11.7	21.8
	2	41	62.2	5.5	8.9	65.4	9.0	13.7
	3	958	63.8	5.9	9.2	52.6	6.0	11.5
CGM	1	958	60.9	9.2	15.1	72.0	14.3	19.9
	2	932	55.4	5.3	9.6	55.8	13.3	23.8
	3	41	62.5	4.6	7.4	50.7	8.9	17.5
BM	1	41	58.5	5.1	8.7	52.2	4.9	9.4
	2	958	57.6	3.6	6.2	65.2	7.6	11.7
	3	932	49.9	4.6	9.3	65.3	13.8	21.1
Mean			58.8	5.6	9.6	59.4	9.9	16.7

[1]Each value is the mean of 18 samples.

[2]CSM = Cottonseed meal; CGM = corn gluten meal; BM = blood meal.

TABLE 5. Ratios of diaminopimelic (DAP) N to total N in bacteria and digesta of duodenal samples, and estimates of percentage of bacterial crude protein in digesta crude protein (BCP/DCP) as affected by protein in three supplements.

Item	Ration[1]			SEM
	CSM	CGM	BM	
DAP N/total N ratios, %				
Bacteria	1.33	1.43	1.45	.19
Digesta	.75	.81	.86	.04
BCP/DCP				
DAP	57.29	59.74	61.54	7.46
^{15}N	61.52[a]	59.43[ab]	54.99[b]	1.05

[a,b]Means within rows with different superscripts differ ($P<.05$).

[1]CSM = Cottonseed meal; CGM = corn gluten meal; BM = blood meal.

and CGM or CGM and BM. As the quantity of undegraded feed protein flowing into the duodenum increases, BCP/DCP should decrease if microbial synthesis is unchanged (5, 16). The lower BCP/DCP for the BM than CSM diet gives greater credence to the ^{15}N compared with the DAP method.

Because of greater variability of the DAP method and an opposite ranking of diets for BCP/DCP for DAP than for ^{15}N, the ^{15}N method was used for further calculations. However, the DAP method did show the BM diet least degradable of the three supplements for true rumen digestibility of CP (43.2, 43,4, and 39.6% for CSM, CGM, and BM, respectively), but treatment differences were lower than for the ^{15}N method (Table 2).

Mean values for duodenal flow of DM, total CP, and MCP for the different protein supplements are in Table 6. There were no differences ($P>.10$) between treatments for flow of DM, total CP and MCP, but the fraction of DM as CP and BCP were different ($P<.05$). On the BM diet, percentage of CP in DM was greater ($P<.05$) than for CGM or CSM. This was due to the numerically lower intake and duodenal flow of total DM coupled with higher flow of CP (kg/d) for the BM diet.

The comparable amount of microbial protein synthesized on the BM compared with the CSM and CGM diets suggests that in this study a lower degradability of the BM protein did not limit N for microbial synthesis. Increased recycling and added urea probably provided sufficient ammonia for microbial synthesis of protein on the BM diet (18). As insurance against N insufficiency for rumen microbes, feeding of urea or other degradable N sources with highly resistant proteins has been beneficial (10).

Increased flow of undegraded feed protein from the BM diet resulted in a slight increase in

TABLE 6. Effect of dietary protein supplement on flow of DM, total CP, and bacterial crude protein (BCP) at the duodenum of cows fed different protein supplements.[1]

Item	Ration[2]			SEM
	CSM	CGM	BM	
DM, kg/d	13.57	11.67	11.30	.73
Total CP				
% of DM	25.00[a]	28.21[a]	32.19[b]	.93
kg/d	3.33	3.24	3.65	.49
Bacterial crude protein (^{15}N method)				
% of DM	15.43[a]	16.85[b]	17.73[b]	.20
kg/d	2.05	1.91	2.02	.32

[a,b]Means within rows with different superscripts differ ($P<.05$).

[1]Nitrogen-15 was used as the microbial marker.

[2]CSM = Cottonseed meal; CGM = corn gluten meal; BM = blood meal.

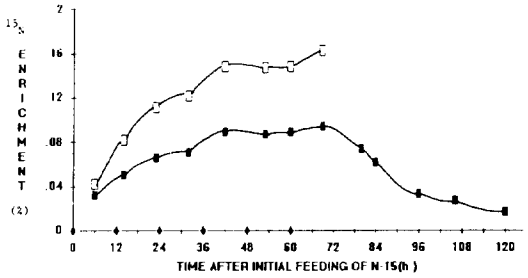

Figure 1. Enrichment patterns of ^{15}N in digesta (■) and bacteria (❐) sampled at the duodenum. Diamond shapes on x-axis indicate feeding times.

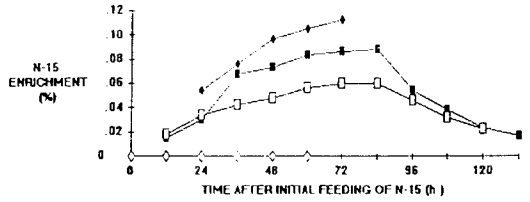

Figure 2. Enrichment patterns of ^{15}N in feces (■), urine (♦), and milk (❐). Diamond shapes on x-axis indicate feeding times.

total protein at the duodenum, although CP intake was slightly higher for CSM. Even greater increases in duodenal protein flow on the BM diet would have been expected had DM intakes been equal.

Efficiency of Microbial Synthesis

Efficiencies of bacterial synthesis (g BCP/100 g fermented organic matter), after organic matter flow to the duodenum was corrected for bacterial synthesis, were 27.4 (± 5.8), 21.7 (± 4.6), and 27.8 (± 6.9) for the CSM, CGM, and BM diets, respectively, with no differences between diets. Stern and Hoover (29) reported efficiencies of 6.3 to 31.6 g CP/100 g digested OM for sheep and cattle across a wide variety of diets. In most cases, values reported by Stern and Hoover (29) were expressed on an apparently digested OM basis and would have been lower if corrected for bacterial synthesis. Increased efficiency of microbial synthesis with decreased degradability of dietary N as observed elsewhere (6, 8) was not shown in this study. The large proportion of concentrate and small particle size of dietary components might have facilitated faster passage of digesta, associated with high efficiencies of microbial synthesis (8, 30, 31).

Enrichment Patterns and Recovery of Nitrogen-15

The ^{15}N enrichment patterns in bacteria, digesta, feces, urine, and milk, averaged for all cows and diets, are in Figures 1 and 2. Greatest enrichment was in the order: bacteria > urine > digesta > feces > milk.

Enrichment in digesta and bacteria increased during the first 40 h and plateaued thereafter to 72 h (Figure 1). Pilgrim et al. (20) reported a fairly steady state in ^{15}N excess in rumen bacteria after 78 h of continuous infusion of the marker into the rumen of sheep. Enrichment patterns for duodenal digesta and bacteria showed parallelism, consistent with an ideal microbial marker. After only 18 to 24 h of ^{15}N feeding, ratios of ^{15}N enrichment in digesta to that in bacteria were relatively constant.

The presence of ^{15}N in milk at 12 h after addition to the diet reflects rapid N utilization in lactating cows (Figure 2). Urea synthesized in the liver from labeled ammonia absorbed from the rumen and from deamination of absorbed microbial amino acids accounted for ^{15}N enrichment of urine.

The appearance of excess ^{15}N in milk demonstrates use of microbial nitrogen for milk protein synthesis, but very early enrichment could have resulted from incorporation of ^{15}N-labelled nonessential amino acids synthesized in the liver from ammonia absorbed from the rumen. Excess ^{15}N in feces would be due mainly to labeled, undigested microbial residues, but recycling of ^{15}N ammonia to the hind gut could also contribute to fecal enrichment.

After 72 h, consumption of ^{15}N-enriched feed ceased, resulting in a subsequent decline of enrichment in all samplings. This decline was rapid, and ^{15}N concentrations approached initial values by 60 h after cessation of feeding, suggesting that the endogenous pool of excess ^{15}N is mostly labile and rapidly depleted.

Incorporation of excess ^{15}N into bacteria ranged from 50 to 83% of intake for individual

TABLE 7. Mean values for ^{15}N incorporation in rumen bacteria and output in urine, feces, and milk during 72-h sampling period for cows fed three protein sources.

Item	Ration[1]			SEM
	CSM	CGM	BM	
Intake, mg/d	1676	1682	1629	
	(% of intake)			
Incorporated into bacteria	65.2	67.6	77.5	6.2
Recovered in feces, urine and milk	73.8	66.3	59.7	4.5
	(% of recovered)			
In feces	43.6	50.3	54.1	3.6
In milk	20.5	17.7	16.2	1.4
In urine	35.9	32.0	29.7	2.7

[1]CSM = Cottonseed meal; CGM = corn gluten meal; BM, blood meal.

cows, while the sum of recoveries in feces, urine and milk ranged from 54 to 78% of intake (Table 7). These recovery estimates are not complete but are based upon ^{15}N appearing in samplings by 72 h after ^{15}N feeding commenced or approximately 12 h after the last feeding of the ^{15}N-enriched diet at cessation of urine collection. However, they do rank the excretion routes in the order: feces > urine > milk. A similar ranking was obtained for cows fed corn silage treated with ^{15}N-ammonium sulfate (28); but urinary excretion was greatest for cows infused with ^{15}N urea into the jugular vein (1).

The numerically high proportion of ^{15}N incorporated into bacteria on the BM diet was probably associated with lower rumen ammonia concentrations than for other diets (24). A given intake of the marker will result in greater enrichment of a smaller pool of ammonia. Because the BM diet was least degradable and N available for microbial synthesis less, there would be greater dependence on that ammonia present for bacterial synthesis.

The ^{15}N method gave less variation and more plausible results than the DAP method for estimating microbial protein flow from three diets of varying ruminal protein degradability fed to lactating dairy cows. Uptake of ^{15}N ammonia by bacteria was more efficient on the least degradable (BM) diet than on CSM or CGM. Ratios of ^{15}N enrichment in digesta and bacteria were constant by about 20 h after feeding of marker commenced and concentrations plateaued at about 40 h.

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