Effect of Replacing Alfalfa Silage with High Moisture Corn on Ruminal Protein Synthesis Estimated from Excretion of Total Purine Derivatives[1]

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ABSTRACT

Twenty-four multiparous dairy cows (eight with ruminal cannulae) were blocked by days in milk and assigned to six balanced 4 × 4 Latin squares with 21-d periods. The four diets, formulated from alfalfa silage plus a concentrate mix based on ground high moisture ear corn, contained (dry matter basis): 1) 20% concentrate, 80% alfalfa silage (24% nonfiber carbohydrate; NFC), 2) 35% concentrate, 65% alfalfa silage (30% NFC), 3) 50% concentrate, 50% alfalfa silage (37% NFC), or 4) 65% concentrate, 35% alfalfa silage (43% NFC). Soybean meal and urea were added to make diets isonitrogenous with equal nonprotein nitrogen (NPN) (43% of total N). Total urine was collected with indwelling Folley catheters for 24 h during each period. There was no effect of diet on urinary creatinine excretion (average 29 mg/kg of BW/d). There were quadratic effects of diet on total urinary ecretion of allantoin, uric acid, and purine derivatives (allantoin plus uric acid), and on ruminal synthesis of microbial N estimated from purine derivatives; maxima occurred at about 35% dietary NFC. Urinary excretion also was estimated with spot urine samples from creatinine concentration and the mean daily creatinine excretion. Daily excretion of allantoin, uric acid, and purine derivatives estimated from spot urine sampling followed the same pattern as that observed with total collection; differences between measured and estimated urine volume were significant only for 35% dietary concentrate. Spot urine sampling appeared to yield satisfactory estimates of purine derivative excretion. Maximal urea N excretion was estimated to occur at about 31% dietary NFC. Milk allantoin secretion increased linearly with concentrate and accounted for 4 to 6% of the total purine derivative excretion. Microbial yield was maximal at 35% dietary NFC, suggesting that this was the optimal level for utilization of dietary NPN from alfalfa silage and other sources.

(**Key words:** alfalfa silage, allantoin, nonfiber carbohydrates, purine derivatives)

Abbreviation key: AS = Alfalfa silage, **HMEC** = ground high moisture ear corn, **NFC** = nonfiber carbohydrates, **PD** = purine derivatives.

INTRODUCTION

Measurement of microbial protein supply continues to be an important area of study in ruminant protein nutrition. Standard in vivo methods to quantify ruminal microbial protein synthesis include using internal markers, such as nucleic acids, or external markers, such as ^{15}N of ^{35}S (4). These procedures also require using animals cannulated in the abomasum or small intestine and estimating digesta flow, which may be laborious and imprecise (4). Using urinary excretion of purine derivatives (**PD**) as a metabolic marker of microbial synthesis in ruminants was proposed by Topps and Elliott (31); however, major progress toward establishing methods relating PD excretion to microbial yield has been made only recently (27). These methods assume that duodenal flow of nucleic acids is essentially all of microbial origin and, after intestinal digestion of the purine nucleotides, absorbed adenine and guanine are catabolized and proportionally excreted in the urine as PD. The PD are excreted primarily as allantoin, but also as hypoxanthine, xanthine, and uric acid (22). Allantoin and uric acid are the only PD present in cattle urine because high xanthine oxidase activity in the blood and tissues converts xanthine and hypoxanthine into uric acid prior to excretion (7).

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Urinary excretion of PD in ruminants can be used to estimate intestinal flow of microbial protein after the quantitative relationship between PD excretion and purine absorption has been determined (9). Thus, recovery of absorbed purines as urinary PD and the purine: total N ratio in ruminal microbes must be defined (34). Methods based on measurement of PD excretion are less invasive than cannulation but require total urine collection. However, it may be possible to simplify measurement of urine output under farm conditions (7). Urinary creatinine excretion is a relatively constant function of BW (28, 34, 35), and it may be possible to use it as a marker for estimating urine output, obviating the need for catheterization and allowing estimation of PD excretion without total urine collection (8). There also is interest in using allantoin concentrations in milk and urine, without total collection, as indicators of intestinal flow of microbial protein (13, 29).

Chen and Gomes (7) state that, to reduce errors due to the variation in urine output, urine should be collected over at least 5 d when PD excretion is being quantified. Urine has been collected over various time periods (11, 12, 14, 34). In trials with catheterized females, collection periods of 5 (28), 3 (34), and 1 to 4 d (36) have been used. Having indwelling catheters in place for several days may cause animal discomfort and depress performance, particularly in lactating and pregnant animals.

The effects on performance of supplementing varying amounts of concentrate to lactating cows fed alfalfa silage was determined in a companion study (38). The present trial was conducted to quantify microbial protein synthesis in the rumen from urinary PD excretion simultaneously in the same cows. Secondary objectives of the trial were to evaluate spot urine sampling for estimating microbial N from total PD excretion and to assess the diurnal variation in p.m. and a.m. urine collections.

MATERIALS AND METHODS

Twenty-four multiparous, lactating Holstein cows, including 8 fitted with ruminal cannulae, were blocked by DIM and assigned to six 4 × 4 Latin squares (two squares of ruminally cannulated cows) with 3-wk periods (total 12 wk). Cows within squares were randomly assigned to dietary treatment sequences. The six 4 × 4 Latin squares were balanced for carryover (i.e., each treatment followed every other treatment once within each square). Treatments were four diets (38), fed as TMR, containing (DM basis) 80, 65, 50, or 35% alfalfa silage (**AS**) as the sole forage plus 20, 35, 50, or 65% of a concentrate mix based on ground high moisture ear corn (**HMEC**). Diets contained, respectively, 24.5, 29.3, 36.2, and 42.8% nonfiber carbohydrate (**NFC**). Other experimental details including description and handling of cows used in the trial, trial design, processing of HMEC, diet composition, feeding protocol, feed sampling and analyses, and weekly adjustment of TMR were as described elsewhere (38). Milk samples from p.m. and a.m. milkings on d 12 and 19 of each period also were deproteinized (2) and stored at –20°C for later analysis.

Samples of whole ruminal contents (100 to 200 ml) were obtained from cannulated cows just prior to feeding (0 h) and 1, 2, 3, 4, and 6 h postfeeding on d 15 of each experimental period. Samples were squeezed through two layers of cheesecloth and pH was measured immediately. Subsamples of ruminal fluid were preserved by addition of 0.2 ml of 50% (vol/vol) H_2SO_4 per 10 ml of ruminal fluid for later analysis of NH_3 and total free AA, and preserved by addition of 5 ml of formic acid per 5 ml of ruminal fluid for later analysis of VFA. These samples were stored at –20°C. Later, ruminal fluid was thawed, centrifuged (15,000 × *g*, 4°C, 15 min) and analyzed for NH_3 and total free AA (3) and for VFA (5). Two additional ruminal samples, obtained at 0 and 4-h postfeeding, were squeezed through two layers of cheesecloth to yield about 750 ml of strained fluid. Particles retained on the cheesecloth were mixed with 250 ml of 0.9% saline, blended, filtrated again, and added to the 750 ml sample (6). These samples were preserved with 10 ml of 40% wt/vol formalin and held at 4°C for approximately 24 h, when one composite sample was prepared for each cannulated cow by mixing equal volumes of fluid from the 0- and 4-h samples. Composites were centrifuged (500 × *g*, 4°C, 20 min), supernatants carefully decanted and then centrifuged again at higher speed (8,000 × *g*, 4°C, 20 min). These pellets were washed once with 0.9% saline and recentrifuged (8,000 × *g*, 4°C, 10 min). The resulting bacterial pellets were dried at 60°C for 48 h (6). Dried bacterial samples were ground and analyzed for DM, ash (1), total N (Leco 2000, Leco Instruments, Inc., St. Joseph, MI) and total purines (32).

Blood was sampled 4 h after feeding from the coccygeal artery or vein of each cow on d 15 (at the time of spot urine sampling) and d 20 (at the time of total urine collection) of each period. Blood was heparinized and stored at 2°C for about 12 h; plasma then was prepared and stored at –20°C for later analysis. Spot urine samples were obtained approximately 4 h postfeeding on d 15 of each period when cows urinated spontaneously; 10-ml aliquots were diluted immediately with 90 ml of 0.036 N H_2SO_4 and stored at –20°C for later analysis. Total urine was collected with indwelling Folley catheters (24 French, 75-ml balloons), which were inserted on d 19 of each period; urine output were measured for

two, 12-h intervals (1700 to 0500 h and 0500 to 1700 h) for 24 h. Fresh containers with 500 ml of 40% H_2SO_4 were attached to catheters at 1700 and 0500 h to acidify (final pH < 3) the urine collected for, respectively, the a.m. and p.m. samples. Just prior to a.m. milking (0400 h), catheters were clamped shut and cows were led to the milking parlow, milked, and immediately returned to their stalls. Fresh containiners were then attached to begin the 0500 to 1700 h collection interval. Catheters remained clamped for about 60 min during the a.m. milking; p.m. milking was done after 1700 h on this day (i.e., after completing the p.m. urine collection). Weights of acidified urine were recorded and 10-ml aliquots were diluted immediately with 40 ml of 0.036 N H_2SO_4 and stored at –20°C for later analysis.

Plasma samples were thawed and deproteinized by mixing 4 volumes of plasma with 1 volume of 25%, wt/vol, trichloroacetic acid and centrifuging (15,000 × *g*, 4°C, 15 min). Milk and urine samples were recentrifuged (15,000 × *g*, 4°C, 15 min) after thawing. Allantoin in milk, plasma, and urine samples was determined by the procedure of Chen and Gomes (7). Urea was determined (30) in deproteinized plasma. Commercial kits were used to analyze these samples for creatinine (No. 555-A; Sigma Chem. Co., St. Louis, MO) and uric acid (Sigma No. 685-50).

Total excretion of creatinine, urea, allantoin, and uric acid for the p.m. and a.m. intervals was computed as the product of the urine volume obtained during each 12-h interval and metabolite concentration. Daily excretion was the sum of p.m. and a.m. excretions. Creatinine clearance (ml/min per kg of BW) was calculated for each 12-h interval as: urine volume × urinary creatinine concentration/(plasma creatinine concentration × BW × 720). One mean daily creatinine excretion rate (29.0 mg/kg of BW per d) was computed with the data from all cows on the trial. Urine volumes used to compute daily excretion of urea, allantoin, and uric acid from spot urine samples were estimated: BW × 29/creatinine concentration (mg/L). Total PD excretion was the sum of allantoin and uric acid excreted in urine plus allantoin excreted in milk. Endogenous PD excretion (mmol/d) was estimated from BW of individual cows as: 0.385 mmol/$BW^{0.75}$ per d (7). Total absorption of microbial purines was calculated as: purine absorption (mmol/d) = (total PD excretion – 0.385 × $BW^{0.75}$)/0.85, where 0.85 is the absorptive efficiency of purines (7). Ruminal synthesis of microbial N was computed as: microbial N (g/d) = (purine absorption × 70)/(0.134 × 0.83 × 1000), where 70 is the N content of purines (mg N/mmol), 0.134 is the mean ratio of purine-N: total-N measured for mixed rumen microbes in the present study and 0.83 is the assumed digestibility of microbial purines (7).

Statistical Analysis

Data were analyzed as a 4 × 4 Latin square, replicated 6 times, with the general linear models procedures of SAS (25). The model included, square, cow-within-square, period, diet, plus diet-by-period interactions. Period-by-treatment was significant for allantoin concentration ($P = 0.05$) in blood plasma sampled during total urine collection and for uric acid excretion ($P = 0.07$) measured using total urine collection so these interactions were included in the models for these variables. No other diet-by-period interactions were significant ($P \geq 0.11$). When significant ($P \leq 0.05$) effects due to diet were detected, mean separations were conducted using Tukey's method ($P = 0.05$). Regressions were obtained with a model that included square, cow-within-square, period and linear and quadratic effects of dietary NFC level. Dietary NFC levels (% of DM) at maximum responses were determined by taking the first derivative of quadratic equations for which the squared terms were significant ($P \leq 0.05$). A repeated measures model was used with the mixed procedure of SAS (25) to analyze ruminal pH and concentrations of NH_3 and total free AA, and excretions of urea, creatinine, allantoin, and uric acid over the 12-h intervals; this model included square, period, diet, day, diet-by-day, and cow (square-by-period-by-diet) interactions and random cow-within-square. A paired test was used to compare observed and estimated urine volumes and excretions of urea, allantoin, uric acid, purine, and microbial N.

RESULTS AND DISCUSSION

There were no differences ($P > 0.05$) in rate of urinary creatinine excretion during the 12-h interval from 0500 to 1700 h; however, there were significant effects due to concentrate level in both creatinine excretion rate and creatinine excreted per unit BW for the 12-h interval from 1700 to 0500 h and overall (Table 1). Overall mean urinary creatinine excretion was about 5% greater ($P < 0.05$) at 50% dietary concentrate, than at 20 and 35% concentrate (Table 1). Average creatinine excretion during the p.m. interval (1700 to 0500 h) also was about 5% greater ($P < 0.05$) than during the a.m. interval (1700 to 0500 h). If it is assumed that there is no diurnal variation in creatinine excretion (8, 35), it may be inferred that urine volume was underestimated during the a.m. interval. Some urine losses may have occurred during the a.m. milking, when the catheters were clamped for about 1 h while cows went to the milking parlor. However, any losses should have been random and similar across diets. Creatinine, which is produced in muscle tissue from the irreversible, nonenzymatic loss of water from creatinine phosphate, is gen-

TABLE 1. Urinary creatinine excretion and clearance obtained for the 12-h intervals from 1700 to 0500 h and 0500 to 1700 h.

Concentrate (% of DM)	Interval		Mean
	1700 to 0500 h	0500 to 1700 h	
Creatinine excretion, g/12 h			
20	9.07^{B}	8.85^{A}	8.96^{B}
35	9.31$^{AB\ a}$	8.64$^{A\ b}$	8.98^{B}
50	9.72$^{A\ a}$	9.15$^{A\ b}$	9.43^{A}
65	9.22^{AB}	8.92^{A}	9.07^{AB}
Mean	9.33^{a}	8.89^{b}	
Creatinine excretion, mg/kg of BW per 12 h			
20	14.43^{B}	14.08^{AB}	14.26^{B}
35	14.70$^{AB\ a}$	13.69$^{B\ b}$	14.19^{B}
50	15.46$^{A\ a}$	14.57$^{A\ b}$	15.01^{A}
65	14.68^{AB}	14.19^{AB}	14.44^{AB}
Mean	14.82^{a}	14.13^{b}	
Creatinine clearance, ml/min per kg of BW			
20	1.51^{A}	1.48^{A}	1.49
35	1.38$^{A\ a}$	1.33$^{A\ b}$	1.36
50	1.39^{A}	1.32^{A}	1.35
65	1.42^{A}	1.38^{A}	1.40
Mean	1.43	1.38	

^{a,b}Means in the same row with different lowercase superscripts differ ($P < 0.05$).
^{A,B}Means in the same column with different uppercase superscripts differ ($P < 0.05$).

erally considered to be excreted in proportion to lean body mass (15). Thus, the somewhat greater creatinine excretion per unit BW on 50% concentrate was surprising and also not consistent with reports that daily creatinine excretion is constant (28, 34, 35). Although statistically significant, the apparent effect of concentrate level on creatinine excretion was numerically small. As discussed below, analysis with the overall statistical model indicated there was no effect ($P > 0.05$) of diet on urinary creatinine excretion. Vagnoni and Broderick (33) reported a significant, 4% increase in creatinine excretion when HMEC was increased from 24 to 40% of dietary DM in cows fed alfalfa silage or hay as the sole forage. In our trial, there were no overall differences ($P > 0.05$) due to concentrate level or sampling interval on creatinine clearance. The average creatinine clearance of 1.41 ml/min per kg of BW was similar to the value of 1.44 ml/min per kg of BW reported for dairy cows (35). Creatinine clearance, a measure of glomerular filtration rate, was not, in the present experiment, affected ($P > 0.05$) by DM intake as was reported for sheep (8).

Urine volume, allantoin, uric acid, and urea N excretions from total collections are in Table 2. Urine volumes excreted during both p.m. and a.m. intervals were higher ($P < 0.05$) when cows were fed 20 and 35% concentrate than 50% concentrate; lowest urine volumes were observed when cows were fed 65% concentrate. On average, urine volume from the p.m. interval (1700

TABLE 2. Urine volume and urinary excretion of allantoin, uric acid, and urea N obtained from total collections for the 12-h intervals from 1700 to 0500 h and 0500 to 1700 h.

Concentrate (% of DM)	Interval		Daily total
	1700 to 0500 h	0500 to 1700 h	
Urine volume, kg			
20	25.3$^{A\ a}$	22.5$^{A\ b}$	47.8^{A}
35	27.5$^{A\ a}$	23.2$^{A\ b}$	50.7^{A}
50	22.2$^{B\ a}$	19.6$^{B\ b}$	41.8^{B}
65	16.5^{C}	15.0^{C}	31.5^{C}
Mean	22.8^{a}	20.1^{b}	
Allantoin excretion, g			
20	29.5^{B}	28.3^{B}	57.8^{B}
35	36.0$^{B\ a}$	32.4$^{AB\ b}$	68.4^{B}
50	45.7$^{A\ a}$	38.9$^{A\ b}$	84.6^{A}
65	35.9$^{B\ a}$	31.9$^{AB\ b}$	67.8^{B}
Mean	36.8^{a}	32.9^{b}	
Uric acid excretion, g			
20	3.22^{C}	2.68^{B}	5.90^{B}
35	4.29$^{AB\ a}$	3.07$^{AB\ b}$	7.36^{AB}
50	4.94$^{A\ a}$	3.87$^{A\ b}$	8.81^{A}
65	3.99$^{BC\ a}$	3.12$^{AB\ b}$	7.11^{AB}
Mean	4.11^{a}	3.18^{b}	
Urea N excretion, g			
20	155.9^{B}	145.8^{A}	301.7^{B}
35	184.4$^{A\ a}$	157.6$^{A\ b}$	342.0^{A}
50	163.1$^{B\ a}$	144.8$^{A\ b}$	307.9^{B}
65	125.9$^{C\ a}$	112.8$^{B\ b}$	238.7^{C}
Mean	157.3^{a}	140.3^{b}	

^{a,b}Means in the same row with different lowercase superscripts differ ($P < 0.05$).
^{A,B,C}Means in the same column with different uppercase superscripts differ ($P < 0.05$).

TABLE 3. Effect of replacing dietary alfalfa silage with concentrate on plasma concentrations of creatinine, allantoin and urea, and milk concentrations of allantoin.[1]

Item	Dietary concentrate (% of DM)				SE	L	Q
	20	35	50	65			
Creatinine spot[2], mg/dl	1.10[a]	1.04[ab]	0.97[b]	1.04[ab]	0.03	0.120	0.227
Creatinine total[2], mg/dl	1.13[a]	0.99[b]	1.08[ab]	1.08[ab]	0.03	0.363	0.385
Allantoin spot, mg/l	30.1[b]	36.9[ab]	36.2[ab]	40.7[a]	2.0	0.001	0.580
Allantoin total, mg/l	32.0[b]	34.5[b]	41.0[a]	43.1[a]	1.5	<0.001	0.868
Urea N spot, mg/dl	23.6[a]	24.2[a]	22.5[a]	18.9[b]	0.5	<0.001	<0.001
Urea N total, mg/dl	21.0[ab]	22.8[a]	20.6[b]	17.3[c]	0.6	<0.001	<0.001
Milk allantoin, mg/l	95.4[b]	99.1[b]	103.2[a]	104.5[a]	1.1	<0.001	0.290

[a,b,c,d]Means in rows with different superscripts differ ($P < 0.05$).

[1]L = P value for linear effect, Q = P value for quadratic effect, SE = standard error.

[2]Concentrations of creatinine, allantoin and urea N in blood plasma samples taken at times of spot urine collection (d 15) or total urine collection (d 20).

to 0500 h) was 14% greater than that from the a.m. interval (1700 to 0500 h). Urine volume was numerically greater for the p.m. than a.m. interval for all concentrate levels; this difference was significant ($P < 0.05$) at all levels except 65% concentrate. Lower volumes for the a.m. sampling interval may be explained by possible urine losses during the a.m. milking. Similar trends for numerically greater excretion during the p.m. interval also were observed for allantoin, uric acid, and urea; these differences were significant ($P < 0.05$) for all diets except 20% concentrate. Vagnoni and Broderick (33) also observed that the urine volume excreted during the 12-h preceding the a.m. milking exceeded that for the 12-h preceding the p.m. milking. Despite the differences observed between p.m. and a.m. collections, the pattern of significance of dietary effects was the same (e.g., for urine volume) or similar (e.g., for allantoin excretion) among p.m., a.m., and total collection periods. Thus, similar statistical inferences would have resulted from use of only 12-h urine collections. Although 12-h collections would have obviated the need to clamp the catheters while cows were taken to the milking parlor, statistical inferences were not identical among treatments over the p.m., a.m., and total collections. Valadares et al. (35) observed no difference in rates of creatinine excretion measured over 12- and 24-h intervals.

Plasma concentrations of creatinine, allantoin, and urea N, measured during spot urine sampling and total urine collection, are in Table 3. There were some differences ($P < 0.05$) in plasma creatinine concentrations among concentrate levels but no linear or quadratic effects ($P > 0.05$) due to dietary concentrate. Plasma allantoin concentrations varied from 30.2 to 40.7 mg/L (spot sampling) and from 32.0 to 43.1 mg/L (total collection). Vagnoni and Broderick (33) found similar plasma allantoin concentrations, ranging from 39.6 to 46.4 mg/l. Plasma allantoin in either sample set increased linearly with dietary concentrate with the highest values obtained for 50 and 65% concentrate; the greatest plasma allantoin concentrations coincided with the highest microbial N yields (Table 4). Milk allantoin concentrations varied from 95.4 to 104.5 mg/L (0.60 to 0.66 m*M*), substantially greater than the 0.11 to 0.18 m*M* reported by Gonda and Lindberg (14). Milk allantoin concentration was greatest ($P < 0.05$) for cows fed diets with 50 and 65% concentrate and, as for plasma allantoin, was linearly related to concentrate level (Table 3). Cows fed 50% concentrate had higher ($P < 0.05$) urinary allantoin excretion than cows fed 20% concentrate (Table 4), suggesting that urinary allantoin excretion could be predicted by milk allantoin concentration. However, Gonda and Lindberg (14) pointed out that changes in milk volume may limit the use of milk allantoin concentration because of dilution effects and allantoin output in milk, rather than milk allantoin concentration, may be a better index of urinary allantoin excretion.

Plasma urea N varied from 18.9 to 24.2 and 17.3 to 22.8 mg/dl, respectively, during spot sampling and total collections and was linearly and quadratically related to concentrate level (Table 4). The approach described by Valadares et al. (35) was used to estimate the plasma urea concentration corresponding to maximum microbial yield. Microbial yield was maximal at 35.4% dietary NFC (Table 5); solving the linear equations relating plasma urea to dietary NFC (Table 5) for urea concentrations corresponding to this level of dietary NFC yielded plasma urea N of 21.7 and 19.7 mg/dl, respectively, for the regressions obtained during spot and total urine collections. These values were higher than the plasma urea N levels of 14.8 and 19 mg/dl reported by Roseler et al. (23) and Valadares et al. (35) for lactating cows. However, it should be noted that all diets in the present study contained very high amounts of NPN (about 43% of total N).

There was no difference ($P > 0.05$) due to concentrate level in urinary creatinine excretion, (Table 4) which averaged 29.0 mg/kg of BW per d (total excretion = 18.2 g/d or 161 mmol/d). This value was higher than the 25.5 and 25.6 mg/kg of BW per d obtained by Valadares et al. (35) in experiments with pregnant and lactating dairy cows, respectively. In other trials with lactating cows, total creatinine excretions of 15.6 to 16.3 g/d (33) and 12.6 to 13.6 g/d (29) were reported. The mean value obtained in the present experiment was used to estimate the daily urinary output from spot urine sampling.

Daily urinary excretions of allantoin, uric acid, and PD were higher ($P < 0.05$) in cows fed 50 than in cows fed 20% concentrate (Table 4); all three traits responded quadratically to concentrate level, with maxima of 35.3 to 35.4% dietary NFC (Table 5). Mean urinary excretion of allantoin varied from 369 to 535 mmol/d and was similar to amounts reported by Vagnoni and Broderick (33) (380 to 492 mmol/d) and Gonda and Lindberg (14) (211 to 571 mmol/d). The proportion of urinary allantoin in total PD excretion was unaffected by concentrate level and ranged from 90.2 to 90.7%. Vagnoni et al. (34) observed a mean of 91.1% allantoin in total urinary PD; proportions of 86.6% (33) and 80 to 85% (7) also have been reported. Daily excretions of allantoin and PD estimated from spot urine sampling generally followed the same pattern as that for total collection (Table 6); however, significant quadratic relationships with dietary NFC were not observed (Table 5).

Urea N excretion ranged from 239 to 342 g/d and declined as dietary concentrate increased from 35 to 65%, indicating better NPN utilization with greater intake of NFC and ruminally fermentable energy. Dietary NPN intake was 295, 350, 361, and 346 g/d for cows fed 20, 35, 50, and 65% of concentrate, respectively; lower NPN intake on 20% concentrate reflected the lower ($P < 0.05$) DM intake on that diet (38). Urea N excretion was related quadratically ($P < 0.001$) to concentrate level (Table 4) and maximum excretion, 335 g/d, was estimated to occur at 30.8% dietary NFC (Table 5). This was higher than the maximum reported by Broderick and Clayton (2). Excretion of urea N, estimated from spot urine sampling, followed the same pattern; maximum excretion was predicted at 32.5% dietary NFC.

Milk allantoin secretion increased linearly with concentrate level ranging from 18.7 to 28.6 mmol/d (Table 4); the greatest mean fell in the range of 28 to 33 mmol/d reported by Vagnoni and Broderick (33). In the present experiment, milk allantoin secretion accounted for 4.2 to 5.7% of the total PD excretion. Chen and Gomes (7) reported that allantoin and uric acid secreted in milk was equivalent to about 5% of that excreted in the urine. Susmel et al. (29) reported that milk allantoin was equivalent to 10.6 to 10.9% of urinary allantoin, while Gonda and Lindberg (14) reported that milk allantoin was equivalent to only 0.63 to 1.34% of urinary allantoin.

TABLE 4. Effect of replacing alfalfa silage with concentrate on daily excretion of urinary creatinine and milk allantoin, and of allantoin, uric acid and urea N measured in total urine collections and estimated from spot urine sampling, and on microbial N synthesized in the rumen as estimated from total excretion of purine derivatives.[1]

	Dietary concentrate (% of DM)						
Item	20	35	50	65	SE	L	Q
Creatinine, mg/kg BW/d	28.6	28.4	30.0	28.9	0.5	0.209	0.281
Milk allantoin, mmol/d	18.7^{d}	22.7^{c}	25.9^{b}	28.6^{a}	0.5	0.001	0.195
Total urine collection							
Allantoin, mmol/d	369.1^{b}	435.2^{ab}	534.8^{a}	428.9^{ab}	31.1	0.005	0.011
Uric acid, mmol/d	35.5^{b}	43.4^{ab}	52.4^{a}	42.3^{ab}	3.7	0.009	0.017
PD[2], mmol/d	423.3^{b}	501.5^{ab}	613.2^{a}	499.8^{ab}	34.1	0.004	0.009
Allantoin, % of PD	90.7	90.2	90.5	90.6	0.5	0.454	0.462
Microbial N, g/d	278^{b}	366^{ab}	419^{a}	335^{ab}	25	0.004	0.009
Urea N, g/d	304^{b}	342^{a}	308^{b}	239^{c}	8	<0.001	<0.001
Spot urine sampling							
Allantoin, mmol/d	405.1^{b}	455.4^{ab}	549.2^{a}	519.0^{ab}	33.8	0.005	0.230
Uric acid, mmol/d	24.6^{c}	33.9^{bc}	45.8^{ab}	52.3^{a}	4.6	<0.001	0.621
PD[2], mmol/d	448.4^{b}	511.9^{ab}	620.8^{a}	599.9^{a}	37.5	0.001	0.244
Allantoin, % of PD	93.5	92.4	91.7	90.3	0.8	0.114	0.360
Microbial N, g/d	297^{b}	344^{ab}	425^{a}	409^{a}	28	0.001	0.245
Urea N, g/d	273^{ab}	293^{a}	293^{a}	256^{b}	10	0.008	0.005

^{a,b,c}Means in rows with different superscripts differ ($P < 0.05$).

[1]L = P value for linear effect, PD = total purine derivatives (allantoin + uric acid), Q = P value for quadratic effect, SE = standard error.

[2]Including milk allantoin excretion. Molecular masses of allantoin and uric acid are, respectively, 158.1 and 168.1 g/mol.

TABLE 5. Significant linear and quadratic regressions on dietary levels of nonfiber carbohydrate (NFC).[1]

Variable (Y)	Type	Equation	(R^2)[2]	Maximum[3]
Plasma and milk concentration				
Allantoin spot[4] (mg/l)	Linear	Y = 19.7 + 0.494 NFC	0.618	. . .
Allantoin total[4] (mg/l)	Linear	Y = 15.9 + 0.654 NFC	0.606	. . .
PUN spot[4] (mg/dl)	Linear	Y = 30.8 – 0.257 NFC	0.702	. . .
PUN total[4] (mg/dl)	Linear	Y = 27.2 – 0.212 NFC	0.584	. . .
Milk allantoin (mg/l)	Linear	Y = 83.8 + 0.505 NFC	0.720	. . .
Urinary excretion (total collection)				
Urine volume (kg/d)	Linear	Y = 73.8 – 0.943 NFC	0.803	. . .
Allantoin (mmol/d)	Quadratic	Y = –1166 + 95.2 NFC – 1.35 NFC^2	0.413	35.3%
Uric acid (mmol/d)	Quadratic	Y = –123 + 9.82 NFC – 0.139 NFC^2	0.402	35.3%
PD (mmol/d)	Quadratic	Y = –1299 + 107 NFC – 1.50 NFC^2	0.421	35.4%
Urea N (g/d)	Quadratic	Y = –314 + 42.1 NFC – 0.683 NFC^2	0.812	30.8%
Microbial N (g/d)	Quadratic	Y = – 998 + 79.0 NFC – 1.11 NFC^2	0.423	35.4%
Urinary excretion (spot sampling)				
Urine volume (kg/d)	Linear	Y = 68.4 – 0.862 NFC	0.560	. . .
Allantoin (mmol/d)	Linear	Y = 267 + 6.56 NFC	0.473	. . .
Uric acid (mmol/d)	Linear	Y = –9.45 + 1.48 NFC	0.430	. . .
PD (mmol/d)	Linear	Y = 265 + 8.56 NFC	0.471	. . .
Urea N (g/d)	Quadratic	Y = –143 + 27.3 NFC – 0.420 NFC^2	0.557	32.5%
Microbial N (g/d)	Linear	Y = 160 + 6.34 NFC	0.472	. . .
Bacterial composition				
OM (%)	Linear	Y = 63.9 + 0.374 NFC	0.771	. . .
N (% DM)	Linear	Y = 6.84 + 0.0622 NFC	0.781	. . .
Ruminal VFA proportion				
Acetate (A)	Linear	Y = 80.9 – 0.641 NFC	0.852	. . .
Propionate (P)	Linear	Y = 3.15 + 0.571 NFC	0.795	. . .
A:P	Linear	Y = 5.76 – 0.0858 NFC	0.831	. . .
Isobutyrate	Linear	Y = 2.09 – 0.0258 NFC	0.888	. . .
Isovalerate + 2-methylbutyrate	Linear	Y = 3.13 – 0.0430 NFC	0.776	. . .

[1]PD = Total purine derivatives (allantoin + uric acid), NFC = dietary NFC (% of DM), and PUN = plasma urea N.

[2]Coefficient of determination.

[3]Dietary NFC content (% of DM) at maximum determined by taking first derivative of quadratic equations, where significant.

[4]Concentrations of allantoin and urea in blood plasma samples taken at times of spot urine collection (d 15) and total urine collection (d 20).

Total PD excretion ranged from 423 to 613 mmol/d and was highest for cows fed 50% concentrate (Table 4); PD excretion on 50% concentrate was greater ($P < 0.05$) than at 20% concentrate. Urinary PD excretions reported by Vagnoni and Broderick (33) had a similar range (473 to 603 mmol/d). There was a quadratic effect of concentrate level on total PD excretion with the maximum predicted at 35% dietary NFC (Table 5). Ruminal microbial N yields, computed from PD excretion using the equation of Chen and Gomes (7), ranged from 278 to 419 g/d (Table 4). Vagnoni and Broderick (33) used the PD excretion technique to estimate microbial N yields ranging from 308 to 362 g/d. Maximum microbial yield also was determined to occur at 35% dietary NFC (Table 5). This was similar to maxima estimated at 37 and 38% dietary NFC for, respectively, DM intake and FCM yield (38). Microbial N yields in the rumen also were computed using NE_L contents (calculated from apparent OM digestibilities) and DM intakes reported earlier for the present experiment (38) using the NRC (21) equation: Microbial N = $-30.93 + 11.45 \times NE_L$ (Mcal/d). This gave estimates of 280, 356, 395, and 420 g/d for, respectively, diets containing 20, 35, 50, and 65% concentrate. Subtracting these estimates from those

TABLE 6. Levels of significance from paired comparisons of daily excretions, determined from total urine collection to those estimated from spot urine sampling, of urine volume, urea, allantoin, uric acid, and total purine derivatives at differing levels of dietary concentrate added to replace alfalfa silage.[1]

	Dietary concentrate (% of DM)			
Daily excretion of	20	35	50	65
Urine volume	0.839	0.034	0.272	0.611
Urea N	0.002	0.001	0.293	0.143
Allantoin	0.111	0.131	0.708	<0.001
Uric acid	<0.001	<0.001	0.143	0.025
Total PD	0.243	0.284	0.850	<0.001

[1]PD = Purine derivatives (allantoin + uric acid).

TABLE 7. Effect of replacing dietary alfalfa silage with concentrate on ruminal pH, ruminal concentration of ammonia, total AA, and VFA, and on molar proportions of ruminal VFA.[1]

	Dietary concentrate (% of DM)						
Item	20	35	50	65	SE	L	Q
pH	6.51[a]	6.29[ab]	6.08[b]	6.08[b]	0.11	0.001	0.195
Ammonia, m*M*	15.20	16.49	16.13	16.23	2.32	0.005	0.011
Total AA, m*M*	1.72[ab]	1.42[b]	1.58[ab]	2.53[a]	0.61	0.009	0.017
Total VFA, m*M*	114.1[b]	129.5[a]	133.0[a]	130.0[a]	5.0	0.089	0.074
Molar proportion, mol/100 mol of total VFA							
Acetate (A)	64.4[a]	62.7[ab]	58.5[b]	52.7[c]	1.2	<0.001	0.190
Propionate (P)	18.2[b]	19.1[b]	22.5[b]	28.6[a]	1.5	0.001	0.093
A:P ratio	3.58[a]	3.30[ab]	2.79[b]	2.00[c]	0.18	<0.001	0.146
Butyrate	11.8	12.5	14.0	14.0	0.5	0.092	0.190
Isobutyrate	1.42[a]	1.38[a]	1.15[b]	0.97[c]	0.04	<0.001	0.482
Isovalerate + 2-methylbutyrate	1.93[ab]	2.04[a]	1.65[b]	1.20[c]	0.10	0.003	0.996
Valerate	2.20	2.28	2.23	2.47	0.14	0.674	0.072

[a,b]Means in rows with different superscripts differ ($P < 0.05$).

[1]L = P value for linear effect, Q = P value for quadratic effect, SE = standard error.

computed from total PD excretion yielded differences of –2, –23, +24 and –85 g/d, respectively. These values were similar for all diets except with 65% concentrate. The large deviation at the highest level of concentrate is interesting and may be attributed to changes in ruminal environment, such as reduced pH, that may depress net yield of microbial protein. Russell et al. (24) reported that net protein synthesis in the rumen declined dramatically when ruminal pH fell below pH 6.2. Average ruminal pH over the 6 h after feeding declined from 6.5 (20% concentrate) to 6.1 on both 50 and 65% concentrate (Table 7). That microbial N yield was 20% lower on 65 than on 50% dietary concentrate suggested that factors in addition to low ruminal pH contributed to depressed microbial protein formation. Ruminal branched-chain VFA were reduced on the diet containing 65% concentrate (Table 7); Hespell and Bryant (17) suggested that inadequate supply of these VFA may depress microbial protein yield.

Daily urine volumes, measured directly or estimated from spot urine sampling, are represented in Figure 1. Mean volumes measured directly were 48.3, 48.4, 41.8, and 31.5 kg/d, and estimated volumes were 47.3, 44.1, 38.8, and 30.0 kg/d for diets containing 20, 35, 50, and 65% concentrate, respectively. With paired tests (Table 6), differences between measured and estimated urine volumes were not significant ($P \geq 0.27$), except for the diet containing 35% concentrate ($P = 0.034$). Urine output decreased linearly with increasing concentrate probably due to dietary K (19), declining, from lowest to highest concentrate level, from 2.12 to 1.23% K (DM basis) (38). Replacing AS, the major dietary source of K, with HMEC and soybean meal which are low in K (21), accounted for this decrease. Although there were differences between observed and estimated excretions of urea N and uric acid, differences in excretions of allantoin and total PD were not signficant for the diets containing 20 to 50% concentrate (Table 6). However, although urine volume was not judged to be different between methods, estimated excretion of allantoin and total PD at 65% concentrate both were different from that measured by total collection. This suggested that variation in catabolite concentration in urine may be a greater problem at higher dietary concentrate. Only one spot urine sample was obtained per period from each cow in the present trial. Increasing the number of spot urine samples over the 24-h day may reduce the effects of variation in urinary catabolite concentrations. Overall, it can be inferred that spot urine sampling may

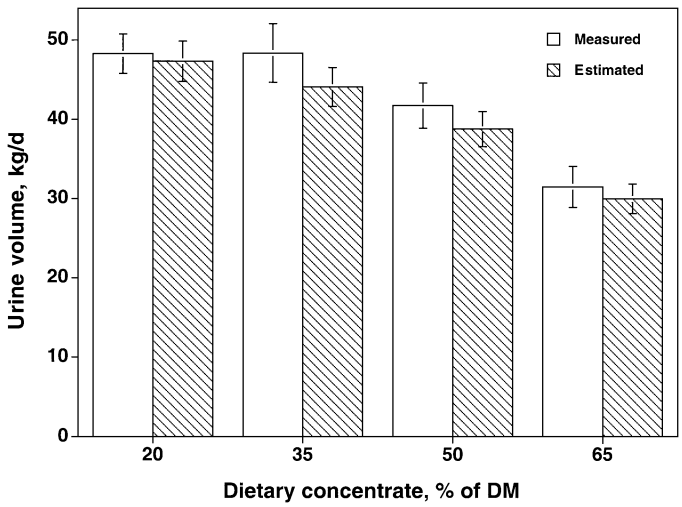

Figure 1. Mean daily urine volumes measured using 24-h total urine collection or estimated from spot urine sampling using creatinine as an output marker. Vertical bars represent ± 1 SE.

TABLE 8. Effect of replacing dietary alfalfa silage with concentrate on composition of bacteria isolated from the rumen.[1]

	Dietary concentrate (% of DM)						
Item	20	35	50	65	SE	L	Q
OM, % of DM	73.2[b]	74.8[ab]	77.1[ab]	80.0[a]	1.5	0.020	0.833
N, % of DM	8.49[b]	8.61[b]	8.87[ab]	9.66[a]	0.20	0.003	0.146
N, % of OM	11.6	11.5	11.5	12.1	0.1	0.063	0.138
RNA, % of DM	7.30	8.06	8.81	8.85	0.56	0.063	0.830
RNA-N, % of total N	12.2	13.5	14.3	13.4	0.7	0.233	0.266

[a,b]Means in rows with different superscripts differ ($P < 0.05$).

[1]L = P value for linear effect, OM = organic matter, Q = P value for quadratic effect, SE = standard error.

be useful for estimating urine volume and excretion of PD and other metabolites for assessing relative difference among dietary treatments in field applications. However, this conclusion is at variance with that of Chen et al. (8), who state that spot urine sampling may not have sufficient sensitivity for comparing dietary treatments in sheep.

The bacterial composition of ruminal contents is in Table 8. There was a linear increase with dietary concentrate in OM and N content (% of DM) of isolated ruminal bacteria. However, there were no differences due to concentrate level in N content, when expressed on OM basis, or in RNA content expressed on a DM basis or as a proportion of total N. Bacterial averages were: total N, 11.7% of OM; RNA, 8.30% of DM; and RNA-N, 13.4% of total N. Concentrations of the same variables in isolated ruminal bacteria were reported to be, respectively: 10.0% of OM, 7.3% of DM, and 13.7% of total N (10); and 8.5% of OM, 8.6% of DM, and 17.6% of total N (37). According to Clark et al. (10), much of the difference in composition of ruminal bacteria may be attributed to differences in techniques used in isolation and analysis. Differences of N content in our study were removed when DM was discounted for ash content. Hespell and Bryant (17) reported differences in composition of bacteria isolated from animals fed at maintenance and ad libitum. The average RNA-N of 13.4% of total N observed in the present trial was higher than the 11.6% used by Chen and Gomes (7) to estimate microbial N yield from urinary PD excretion when ruminally cannulated animals were not available.

There was no effect on ruminal pH due to time after feeding in cows fed 20 and 35% concentrate; however, depressed ruminal pH on 50 and 65% concentrate (Figure 2a) reflected the greater NFC intakes on those diets. The pH of 5.86 obtained at 6 h on 50% of concentrate was lower ($P < 0.05$) than that at 0 h (immediately before feeding) and 1 h postfeeding. When cows were

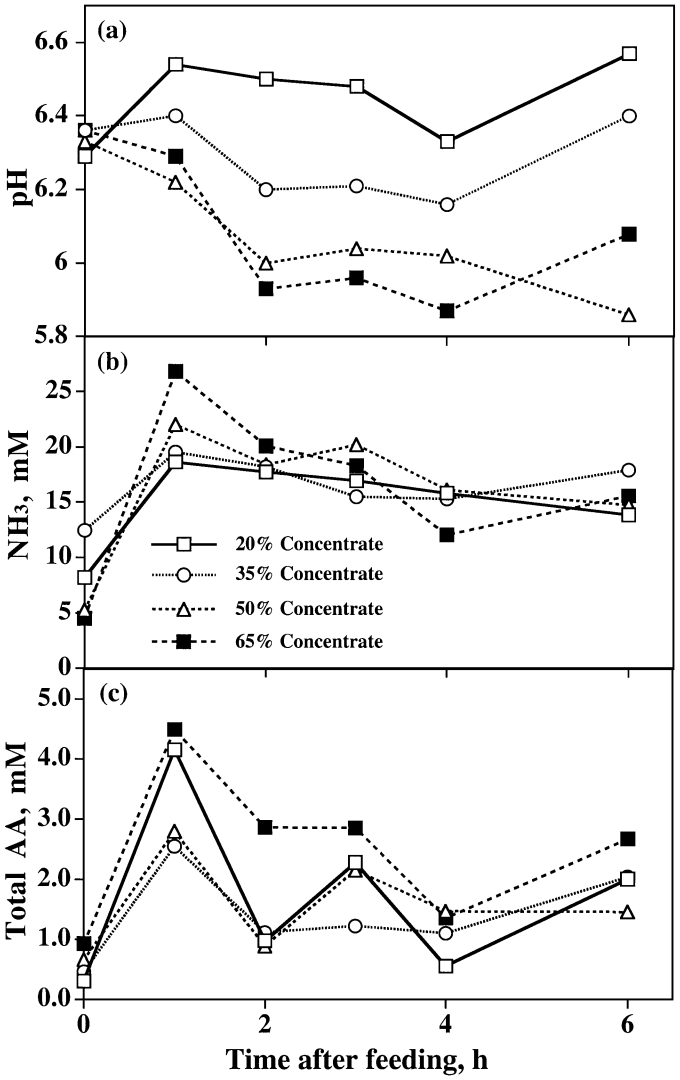

Figure 2. Mean hourly a) pH, b) NH_3 concentration, and c) total AA concentration in the rumen of cows fed diets with all forage from alfalfa silage and 20, 35, 50 or 65% of dietary DM as a concentrate mix based on ground high moisture ear corn.

fed 65% concentrate, the 5.93 pH observed at 2 h postfeeding was lower ($P < 0.05$) than that at 0 and 1 h postfeeding but did not differ from other times. Mean ruminal pH of cows fed 20% concentrate was higher ($P < 0.05$) than at 50 and 65% dietary concentrate (Table 7) and remained higher than on the other three diets over the 6 h after feeding (Figure 2a). Ruminal pH on 35% dietary concentrate was intermediate. Mould et al. (20) described a biphasic effect of ruminal pH of fiber digestion whereby pH reduction from 6.8 to about 6.0 resulted in moderate depression, but pH reduction below 6.0 caused severe inhibition of fiber digestion. According to Hoover (18), reductions of ruminal pH to the range of 5.8 to 6.2 that are cyclic and of short duration cause only moderate, transient depressions in fiber digestion. However, further pH reductions for longer periods cause washout of the ruminal organisms associated with fiber digestion, severely reducing fiber and OM digestion and microbial protein yield (18). There was a linear reduction in apparent NDF digestibility observed in the present experiment with increasing dietary concentrate (38). Despite the feeding of buffers in all diets (38), ruminal pH after feeding fell below 6 at only one time (6 h after feeding) on 50% dietary concentrate, but was below pH 6 at three time-points on 65% concentrate (Figure 2a). However, these small differences in ruminal pH pattern likely do not explain the 20% depression in microbial N yield (335 versus 419 g/d) observed on 65% versus 50% concentrate because overall mean pH on these two diets was identical (Table 7).

Ruminal NH_3 was very high at all times after feeding (Figure 2b) and there were no differences among diets in mean concentration (Table 7). High ruminal NH_3 is not surprising in view of the high dietary CP levels of 19.5 to 20.1%, with 43% of the CP equivalent coming from NPN (38). There were differences in total AA concentrations after feeding (Figure 2c); overall, total AA tended to be higher on the 65% concentrate diet (Table 7), the diet that contained the greatest amount of solvent soybean meal (38). Peak ruminal NH_3 and total free AA concentrations for all diets occurred 1-h postfeeding (Figure 2). This was in accordance with results from other trials in which cows were fed AS diets; results reflect the extensive ruminal degradation of CP in AS, soybean meal and urea, as well as the large amount of peptides and free AA present in AS (33). For all diets and times after feeding, ruminal NH_3 concentration greatly exceeded the 3.6 m*M* suggested by Satter and Slyter (26) as the minimum necessary to maintain ruminal bacterial growth. It may be inferred from these high ruminal NH_3 concentrations that energy fermentation was not sufficient to stimulate utilization of all of the CP degraded in the rumen on these diets.

Ruminal concentration of total VFA was unaffected by concentrate level (Table 7). Concentrations of VFA represent a balance between production and disappearance (33), and any increase in production rate that accompanied increasing concentrate intake may not be apparent from VFA concentrations due to the effects of ruminal volume and VFA absorption. Molar proportions of acetate decreased linearly and propionate increased linearly, and acetate to propionate ratio declined linearly, with decreased AS and increased concentrate in the diet (Table 7). Changes in ruminal VFA pattern are typical of replacing dietary forage with concentrate when more NFC and less fiber are fermented in the rumen (16). These alterations in ruminal VFA were consistent with the reduced milk fat content and yield observed on 65% dietary concentrate in this trial (38). Molar proportions of isobutyrate and isovalerate plus 2-methylbutyrate also declined linearly with decreasing AS and increasing concentrate (Table 7). These branched-chain VFA are formed from microbial catabolism of branched-chain AA and their concentrations likely would be directly related to the intake of free AA and peptides in AS NPN. In this trial, dietary NPN was maintained at about 43% of total N by adding more urea as concentrate was increased (38); urea hydrolysis, of course, would not give rise to branched-chain VFA. Molar proportions of butyrate and valerate were unaffected by diet (Table 7). The equations describing the significant linear relationships between ruminal VFA and dietary NFC are in Table 5.

CONCLUSIONS

Purine derivative excretion, measured in 24-h total urine collections, gave satisfactory estimates of microbial N yields in lactating cows fed varying levels of dietary concentrate to replace AS. A single spot urine sample from each cow in each period to quantify urine volume based on creatinine excretion gave nearly the same estimates of urinary PD output and, hence, microbial N yield, as total urine collection. Also, urinary collection periods of only 12 h showed promise for estimating total daily urine excretion; however, further research is necessary to confirm this result. Maximum microbial N yield was observed at 35% dietary NFC, suggesting that this was the optimum level for feeding NFC from a HMEC-based concentrate to maximize utilization of the NPN in AS.

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