

Synthesis of Microbial Protein in Ruminally Cannulated Cows Fed Alfalfa Silage, Alfalfa Hay, or Corn Silage¹

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ABSTRACT

Six ruminally cannulated cows were used in an experiment with a 3 × 3 Latin square design. Three all forage diets—alfalfa silage, alfalfa hay, or corn silage plus 2.2% urea (DM basis)—were fed for ad libitum intake four times daily. The microbial protein marker ¹⁵NH₃ and the liquid marker Cr-EDTA were infused continuously into the rumen for 72 and 48 h, respectively; the solid marker, Yb-labeled forage, was dosed into the rumen twice daily for 60 h. Pool sizes of ruminal NAN were determined by emptying the rumen. Proportions of bacterial N formed from NH₃ were 57, 46, and 82% for the alfalfa silage, alfalfa hay, and corn silage diets, respectively. For all diets, flows of microbial NAN with the liquid and solid phases were about equal. Although feed NAN in the liquid pool was only 12% of ruminal feed NAN, 30% of feed NAN that escaped the rumen flowed with the liquids. Flow of microbial NAN was highest for corn silage (243 g/d) and lowest for alfalfa hay (212 g/d); microbial NAN represented 50% (alfalfa silage and hay) and 76% (corn silage) of total NAN flow. Proportions of NAN intake that were degraded in the rumen were 61, 56, and 57% for alfalfa silage, alfalfa hay, and corn silage (without urea N), respectively; these values were lower than those reported by the NRC. Total flows of NAN from the rumen were 472, 424, and 321 g/d for the alfalfa silage, alfalfa hay, and corn silage diets, respectively. Use of liquid (Cr-EDTA) and solid (Yb) markers to compute the rate of passage of microbial protein proved to be less variable than regression of ¹⁵N enrichment of bacterial NAN over time.

(**Key words:** ruminal synthesis of microbial proteins, nitrogen-15, alfalfa forage, corn silage)

Abbreviation key: AH = alfalfa hay, AS = alfalfa silage, CS = corn silage, LP = liquid phase of ruminal contents, SP = solid phase of ruminal contents.

INTRODUCTION

Microbial protein synthesis plus ruminal protein escape are the two basic sources of α-amino N supplied to ruminants. Quantitative estimation of both is important for proper diet formulation for dairy cows (26). It should be possible to estimate the microbial protein synthesis in, and the dietary protein escape from, the rumen of cows equipped with ruminal cannulas only. Walker and Nader (36) used this approach by labeling microbial protein with ³⁵S and using the decline in bacterial ³⁵S to estimate the flow rate of bacterial protein. Fluid markers, polyethylene glycol or Cr-EDTA, were used to estimate the size of the ruminal pool of microbial protein (36). Reliability of this approach might have been limited because separation was not made between ruminal bacteria associated with fluid and particulate phases. Hristov et al. (14) attempted to overcome this problem by estimating the pool size of each population from separate analyses of labeling patterns of bacteria in the fluid and particulate phases. The stable isotope ¹⁵N has been used frequently as a microbial marker to distinguish between microbial and feed protein in the rumen and to quantify the microbial protein leaving the rumen (21, 32, 33). However, ¹⁵N usually has been used with animals fitted with cannulas in the rumen plus abomasum or duodenum.

Alfalfa silage (AS), alfalfa hay (AH), and corn silage (CS) are major sources of forage and protein for dairy cows. Differences in milk protein yields (4) suggested that ruminal escape of dietary protein and ruminal formation of microbial protein in cows fed these forages may differ substantially from values reported by the NRC (26). The objective of this study was to estimate production of microbial protein and protein degradability of AS, AH, and CS diets in the rumen of cows fitted with ruminal cannulas only.

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MATERIALS AND METHODS

Methodological Considerations

Microbial protein produced in the rumen and feed protein escaping from the rumen were estimated by quantifying the following in both the liquid phase (LP) and the solid phase (SP) of ruminal contents: 1) proportions of microbial NAN in total NAN, 2) passage rates, and 3) pool sizes of total NAN. The major assumptions made in this trial were that 1) ruminal microbial protein reached a plateau for enrichment after 48 h of continuous infusion of $^{15}\text{NH}_3$ (i.e., although ^{15}N enrichment of the NH_3 pool altered with time after feeding, there was no net change in the 10-h mean ^{15}N enrichment of microbial protein after 48 h); 2) passage rates of Cr-EDTA and Yb-labeled feed particles of each diet represented, respectively, passage rates of LP and SP; and 3) although ^{15}N enrichments of bacteria and protozoa N were different, these differences did not greatly influence overall estimates of total flow of microbial NAN.

Cows and Feeding

Six multiparous cows, fitted with 10-cm ruminal cannulas (Bar Diamond Inc., Parma, ID), weighing ($\bar{X} \pm \text{SD}$) 619 ± 42 kg at 198 ± 15 DIM, and yielding 23 ± 3 kg/d of milk, were blocked into two groups by DIM and milk yield and were randomly allotted to treatment sequences in two 3×3 Latin squares. The two squares were arranged such that each diet followed each other diet an equal number of times during the trial. First-cutting alfalfa was mowed, field-wilted, and harvested from the same fields as AS at about 40% DM or as AH in small rectangular bales at about 85% DM. Neither AS nor AH was rained on. The AS was chopped to a theoretical length of 1.0 cm and ensiled in a concrete stave tower silo without additives. The AH was stored under shelter and was chopped to a 4.0-cm theoretical length of cut when fed. The CS also was chopped to a 1.0-cm theoretical length of cut and ensiled without additives in a concrete stave tower silo. At the time of feeding, CS was mixed with 2.2% urea (DM basis) and fed as a TMR. The compositions of the three forages and diets are presented in Table 1. Diets were fed for ad libitum intake four times per day (0400, 1000, 1600, and 2200 h); the daily amount of feed offered was adjusted to yield about 5% orts, which were measured once per day. Weekly composites of AS, chopped AH, CS TMR and and orts were collected from daily samples of about 0.5 kg and stored at -20°C . Diets were fed for three 4-wk periods (total 12 wk); marker

TABLE 1. Diet and forage composition.^{1,2}

Item	AS	AH	CS ³
AS, %	100
AH, %	...	100	...
CS, %	97.8
Urea, %	2.2
Composition			
Forage DM, %	38.8	86.2	36.2
CP, % of DM	20.9	18.3	14.2
NE _L , ⁴ Mcal/kg of DM	1.35	1.29	1.59
NDF, % of DM	44.9	47.9	39.2
ADIN, % of Total N	6.2	6.4	4.1
Extract pH	4.7	...	3.9
Urea NPN, % of total N	46.0
NH ₃ N, % of Total N	7.8	1.3	3.6
Free AA N, ⁵ % of Total N	29.2	8.0	16.9
NPN, ⁶ % of Total N	51.9	14.0	71.0
Unidentified NPN, ⁷ % of total N	14.9	4.7	4.5
Estimated UIP, ⁸ % of total N	45	39	21

¹AS = Alfalfa silage, AH = alfalfa hay, and CS = corn silage.

²Trace-mineralized salt containing 5.4 g/kg of Mn, 5.4 g/kg of Zn, 3.4 g/kg of Fe, 1.4 g/kg of Cu, 0.08 g/kg of I, 0.06 g/kg of Se, and 0.02 g/kg of Co was available for free choice.

³Corn silage alone contained (DM basis) 8.2% CP, 46.5% NPN (total N basis), and 37% estimated undegraded intake protein.

⁴Values for NE_L computed from NDF for AS, AH (23), and CS (NE_L = 2.394 - 0.0193 × NDF; D. R. Mertens, 1994, personal communication).

⁵Free AA N = total AA × (40 mg of N/mmol of total AA) (5).

⁶Proportion of total N soluble in 10% (wt/vol) TCA (24).

⁷Unidentified NPN as [NPN - (NH₃ N + free AA N)].

⁸Ruminal undegraded intake protein estimated with an inhibitory in vitro system from net release of NH₃ and total AA, assuming a ruminal passage rate of 0.06/h (5).

dosing and sampling were done during the last week of each period. Cows had free access to water during the trial; water intake was recorded daily using flow meters. Milk yield was recorded at all daily a.m. and p.m. milkings. Milk was sampled at one a.m. and one p.m. milking midway through wk 2 and 3 of each period and was analyzed for fat, protein, lactose, and SNF contents by infrared analysis (Wisconsin DHI Cooperative, Madison). Cows were weighed on 3 consecutive d at the start of the trial and at the end of each period.

Dosing of the Markers and Ruminal Sampling

Microbial protein was distinguished from feed protein with $^{15}\text{NH}_3$ used as a microbial marker; Cr-EDTA and Yb, respectively, were used as flow markers for LP and SP. A solution containing 1.25 g/L of ($^{15}\text{NH}_4$)₂SO₄ (73 atom % ^{15}N ; Monsanto Research Corp., Miamisburg, OH) in distilled water was infused continuously into the rumen for 72 h at a rate of

1200 ml/d per cow (0.25 g of ^{15}N /d per cow) using a peristaltic pump. The infusion was started at 1000 h on d 1 and stopped at 1000 h on d 4. On d 2 at 1000 h, a priming dose of Cr-EDTA solution (3) equal to one-half of the daily dose of Cr-EDTA (600 ml) was added to the ventral rumen. Then Cr-EDTA was infused into the rumen at 1200 ml/d per cow for 48 h (2.7 g of Cr/d per cow) by a peristaltic pump, starting on d 2 at 1000 h and stopping at 1000 h on d 4.

The Yb marker was adsorbed to feed material prepared from each forage. Grain, cob, and stalk particles >2 to 3 cm were removed from CS; AH that had been chopped for feeding was sieved, and the material remaining on a 4-mm screen was used for treatment. The AS was used as fed. Water-soluble DM was extracted from 4.2-kg, 2.1-kg, and 5.4-kg (as-fed basis) samples of AS, AH, and CS, respectively, by soaking samples in distilled water for 24 h at 39°C and then washing them thoroughly with tap water through a polyester fabric with 1.15-mm pores. [Faichney (12) found that feed particles >1.18 mm were selectively retained in the rumen of cattle.] In 15-L buckets, 156 g of $\text{YbCl}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 10 L of distilled water previously acidified to pH 5 with HCl so that the final pH of the Yb solution was neutral. Particles of each forage retained on the fabric were labeled by soaking for 48 h in the neutral Yb solution at 25°C. After soaking, loosely bound Yb was removed by washing thoroughly with tap water through the 1.15-mm fabric, soaking for 1 h in an acetic acid solution (pH 4.5 to 5.0), and then washing again with tap water through the 1.15-mm fabric. Labeled forage samples were divided into 36 (3 periods \times 2 cows \times 6 doses) equal doses of 100 g of DM; final Yb concentrations achieved were 95.2 (AS), 74.4 (AH), and 22.3 (CS) mg/g of DM. Treated AH was dried at 60°C overnight in a forced-air oven. The two labeled silage types were stored wet at 4°C until dosing. Cows were dosed with 100 g of DM of their respective Yb-labeled forage material at 0800 and 2000 h for 60 h (i.e., 6 doses per cow per period) beginning at 0800 h on d 1 and ending at 0800 h on d 4. Markers were given by removing about 5 kg of solids from the dorsal rumen, mixing the solids with one dose of labeled feed, and then returning the mixture to the rumen.

Three samples of ruminal contents were taken on d 1, just before starting the marker dosing, to determine background ^{15}N enrichment and concentrations of Cr and Yb. On d 3, six samples were taken at 2-h intervals beginning at 1000 h and ending at 2000 h (48 to 58 after starting $^{15}\text{NH}_3$ infusion); these samples were analyzed for ^{15}N enrichment of NAN in bacteria, protozoa, and whole ruminal contents. Sam-

ples consisted of 300 ml of whole ruminal contents taken from each of four locations: the cranial and caudal regions of both the ventral and dorsal rumen (total 1200 ml). After mixing, two 500-ml subsamples were retained. One subsample was squeezed through two layers of cheesecloth; bacteria loosely attached to solids were dislodged by manually shaking the residue from this subsample in a 1-L jar with 500 ml of cold McDougall's buffer and then squeezing the subsample again through two layers of cheesecloth (11). The two cheesecloth filtrates were mixed and held in 500-ml graduated cylinders at 39°C for 30 to 40 min to separate bacteria and protozoa (30). Two 50-ml samples were taken using a vacuum pipet, one from the bottom (presumably enriched with protozoa) and one from the middle (presumably enriched with LP and loosely attached bacteria) of the sample column in the cylinder. These samples were preserved with formalin (1%, wt/vol, final concentration) and stored at 4°C until processed the next day. Protozoa were isolated from the lower fraction by centrifugation at $551 \times g$ at 4°C for 10 min (Beckman J2-21; Beckman Instruments, Palo Alto, CA). Bacteria were isolated from the middle fraction by differential centrifugation. Protozoa plus feed particles were sedimented at $551 \times g$ at 4°C for 10 min and discarded; supernatants were centrifuged at $31,000 \times g$ at 4°C for 25 min to allow sedimentation of bacteria. Protozoal and bacterial pellets were washed with formalin and saline (1%, vol/vol, formalin plus 0.9%, wt/vol, NaCl), recentrifuged ($31,000 \times g$ at 4°C for 25 min), resuspended in formalin and saline, and stored at -20°C. Samples were later thawed, recentrifuged ($31,000 \times g$ at 4°C for 25 min), and then freeze-dried. Bacterial and protozoal samples were treated with saturated K_2CO_3 to remove NH_3 (25) and analyzed for ^{15}N enrichment of NAN by isotope ratio mass spectrometry (15).

The second set of 500-ml subsamples that were taken at 48 to 58 h was pressed through a polyester fabric with a 0.105-mm pore size at 1034 kPa using a mechanical press to separate LP [solubles and small feed particles thought to pass from the rumen with the liquids (12)] from SP. Samples of LP and SP were collected quantitatively, weighed, freeze-dried, ground through a 1-mm screen, treated with K_2CO_3 to remove NH_3 , and analyzed for ^{15}N enrichment of NAN as described. Proportions of microbial NAN in LP and SP NAN were computed:

$$\text{microbial NAN}_{\text{LP}} (\text{percentage}) = \left(\frac{^{15}\text{N NAN}_{\text{LP}}}{^{15}\text{N bacterial NAN}} \right) \times 100$$

and

$$\text{microbial NAN}_{\text{SP}} \text{ (percentage)} = \left(\frac{^{15}\text{N NAN}_{\text{SP}}}{^{15}\text{N bacterial NAN}} \right) \times 100$$

where $^{15}\text{N NAN}_{\text{LP}}$ and $^{15}\text{N NAN}_{\text{SP}}$ are mean ^{15}N enrichments (atom percent excess) in LP and SP, respectively, from the six samples taken at 48 to 58 h, and $^{15}\text{N bacterial NAN}$ is the mean ^{15}N enrichment (atom percent excess) in the isolated bacterial NAN from the same six samples.

Marker infusions were stopped at 1000 h on d 4; 11 samples were taken for Cr, Yb, and ^{15}N kinetics analyses, starting with a 0-h sample at 1000 h (just before feeding) and then at 2, 4, 6, 8, 11, 14, 17, 20, 23, and 26 h (1200 h on d 5). The LP and SP were separated at 1034 kPa as described, freeze-dried, and then ground through a 1-mm screen. Samples (1 g) were ashed at 500°C for 16 h and then solubilized in 10 ml of concentrated HCl and a solution of 0.6% (wt/wt) LiOH to a final mass of 100 g; concentrations of Cr and Yb were analyzed by direct current plasma emission spectroscopy (9) (SpectraSpan V; Fison Instruments, Valencia, CA). A preparation enriched with LP bacteria was isolated for ^{15}N analysis as described by Craig et al. (11), except that squeezed solids were washed four times with McDougall's buffer, and filtrates were centrifuged ($551 \times g$ at 4°C for 10 min). Bacterial pellets were isolated from the supernatants, and the ^{15}N enrichment of bacterial NAN was determined as described. Ruminal fluid

obtained at these 11 time points also was preserved with 1 ml of 50% (vol/vol) $\text{H}_2\text{SO}_4/50$ ml and was analyzed for NH_3 N and total AA (6), for VFA [(7); Varian Vista 6000 GC; Varian Instruments, Sugarland, TX], and for ^{15}N enrichment of NH_3 N (15).

Emptying of the Rumen

Ruminal pool sizes of NAN were estimated by emptying the rumen of each cow twice following the 5-d infusion period: just before feeding at 1000 h on d 6 and at 3 h after feeding at 1300 h on d 8 (1 and 3 d after stopping marker infusion). Weights of total ruminal contents were recorded. Total ruminal contents were mixed intensively in a large cart using a hoe; five 500-ml subsamples were taken on d 6, and three 500-ml subsamples were taken on d 8. Subsamples were separated into LP and SP as described, and the weights of the LP and SP compartments were recorded. The DM content was determined by freeze-drying; samples were treated with K_2CO_3 , and Kjeldahl analysis was used to determine pool sizes of NAN according to the procedures of the AOAC (2).

Other Analyses

Blood was sampled 4 h after feeding from the coccygeal artery or vein on 2 consecutive d during the sampling period. Blood was heparinized, held at 2°C

TABLE 2. Dietary nutrient intakes and digestibilities.¹

Item	AS	AH	CS	SE	<i>P</i> > <i>F</i> ²
Intake					
Water, L/d	81.1 ^b	103.1 ^a	57.2 ^c	2.8	<0.001
DM, kg/d	19.1 ^a	17.1 ^{ab}	16.0 ^b	0.4	0.058
OM, kg/d	17.1 ^a	15.4 ^{ab}	14.9 ^b	0.4	0.088
Total N, g/d	646 ^a	517 ^b	370 ^c	11	0.007
NAN, ³ g/d	591 ^a	493 ^b	183 ^c	11	0.002
NE _L , Mcal/d	25.9 ^a	22.0 ^b	25.3 ^a	0.5	0.063
RDOM, ⁴ kg/d	7.4 ^{ab}	6.5 ^b	7.9 ^a	0.9	0.075
Apparent digestibility, ⁵ %					
DM	56.4 ^b	54.8 ^b	68.9 ^a	0.9	<0.001
OM	56.6 ^b	55.3 ^b	69.4 ^a	0.8	<0.001
NDF	41.7 ^b	40.8 ^b	48.3 ^a	1.4	0.002
ADF	45.1	43.0	44.7	1.5	0.582
CP	66.7 ^b	66.5 ^b	74.6 ^a	1.2	<0.001

^{a,b,c}Means within the same row without a common superscript differ (*P* < 0.05).

¹AS = Alfalfa silage, AH = alfalfa hay, and CS = corn silage.

²Probability of a dietary effect.

³Based on K_2CO_3 treatment of freeze-dried samples. Urea N was not included in NAN content of the corn silage diet.

⁴Ruminally digested OM (RDOM; percentage) = apparent OM digestibility \times 0.76 (18).

⁵Apparent digestibilities determined by using indigestible ADF as an internal marker (8).

TABLE 3. Mean ruminal ^{15}N enrichments of NAN in liquid and solid phases, isolated bacteria and protozoa, and estimated size and origin of microbial NAN pools.^{1,2}

Item	AS	AH	CS	SE	$P > F^3$
Bacterial NAN ^{15}N , atom % excess	0.045 ^c	0.052 ^b	0.057 ^a	0.002	<0.001
Liquid NAN ^{15}N , atom % excess	0.028 ^c	0.033 ^b	0.048 ^a	0.001	<0.001
Liquid microbial NAN, ⁴ % total N	63.1 ^b	63.3 ^b	86.3 ^a	2.1	<0.001
Solid NAN ^{15}N , atom % excess	0.018 ^c	0.021 ^b	0.038 ^a	0.001	<0.001
Solid microbial NAN, ⁴ % total N	40.9 ^b	39.8 ^b	67.9 ^a	1.4	<0.001
Protozoal NAN ^{15}N , atom % excess	0.025 ^c	0.032 ^b	0.042 ^a	0.001	<0.001
Protozoa:bacteria ^{15}N ratio	0.54 ^c	0.61 ^b	0.74 ^a	0.01	<0.001

^{a,b,c}Means within the same row without a common superscript differ ($P < 0.05$).

¹AS = Alfalfa silage, AH = alfalfa hay, and CS = corn silage.

²Mean ^{15}N enrichments from 48 to 58 h after beginning of $^{15}\text{NH}_3$ infusion.

³Probability of a dietary effect.

⁴Microbial NAN in liquid or solid phase NAN (percentage) = (^{15}N enrichment of liquid or solid phase NAN/ ^{15}N enrichment of bacterial NAN) \times 100.

for 4 h until plasma was prepared, deproteinized, and stored at -20°C until analyzed for glucose and urea (4). One fecal grab sample was taken per cow on d 1 and 3 during the infusions; samples were dried at 60°C for 72 h and then ground through a 1-mm screen (Wiley mill; Arthur H. Thomas, Philadelphia, PA). Feces and composite forage samples were analyzed for total N, DM, OM (2), NDF, ADF, and ADIN (34). Indigestible ADF was determined as the residual ADF after 144 h of in vitro incubations (8); apparent total tract digestibilities of nutrients were estimated using indigestible ADF as an internal marker (8). Silage extracts were prepared from composite samples in distilled H_2O (24) and analyzed for pH, NH_3N , total free AA (6), and NPN (24).

Results were analyzed statistically as a replicated 3×3 Latin square using the general linear models

procedures of SAS (35). The model included square, cow within square, period within square, replicate (when appropriate), diet, period by diet interaction, hour by diet interaction (after stopping marker infusions), and weighting (measurements of the ruminal pool). When the period by diet or hour by diet interactions were not significant ($P \geq 0.10$), those data were pooled with the residual effects. When diet effects were significant ($P < 0.05$), mean separation was by least significant difference.

RESULTS AND DISCUSSION

Milk yield, which averaged 17.2 kg/d, was low because cows were in late lactation and were fed all forage diets. Neither milk output nor composition differed significantly among diets. Cows on the three

TABLE 4. Pool sizes of wet contents, DM, and NAN in liquid and solid phases in the rumen.^{1,2}

Item	AS	AH	CS	SE	$P > F^3$
Whole ruminal contents (wet), kg	82.7 ^a	81.9 ^a	75.4 ^b	2.9	0.003
Liquid phase					
Wet contents, kg	42.5 ^{ab}	43.3 ^a	37.9 ^b	1.7	<0.001
% DM	2.85 ^a	2.73 ^b	2.87 ^a	0.03	0.005
kg of DM	1.21 ^a	1.18 ^a	1.09 ^b	0.06	0.034
NAN, % of DM	3.94 ^a	3.97 ^a	3.70 ^b	0.04	<0.001
NAN, g	47.6 ^a	47.3 ^a	40.8 ^b	3.3	0.011
Solid phase					
Wet contents, kg	40.2	38.5	37.4	2.1	0.171
% DM	26.22	26.06	26.41	0.24	0.578
kg of DM	10.54	10.05	9.89	0.39	0.068
NAN, % of DM	2.16 ^a	2.12 ^a	1.48 ^b	0.02	<0.001
NAN, g	229.9 ^a	212.4 ^a	147.3 ^b	9.9	<0.001
Total ruminal NAN, g	277.5 ^a	260.1 ^a	188.1 ^b	9.8	<0.001

^{a,b}Means within the same row without a common superscript differ ($P < 0.05$).

¹AS = Alfalfa silage, AH = alfalfa hay, and CS = corn silage.

²Means from ruminal dumping; measurements were taken at 0 and 3 h after feeding.

³Probability of a dietary effect.

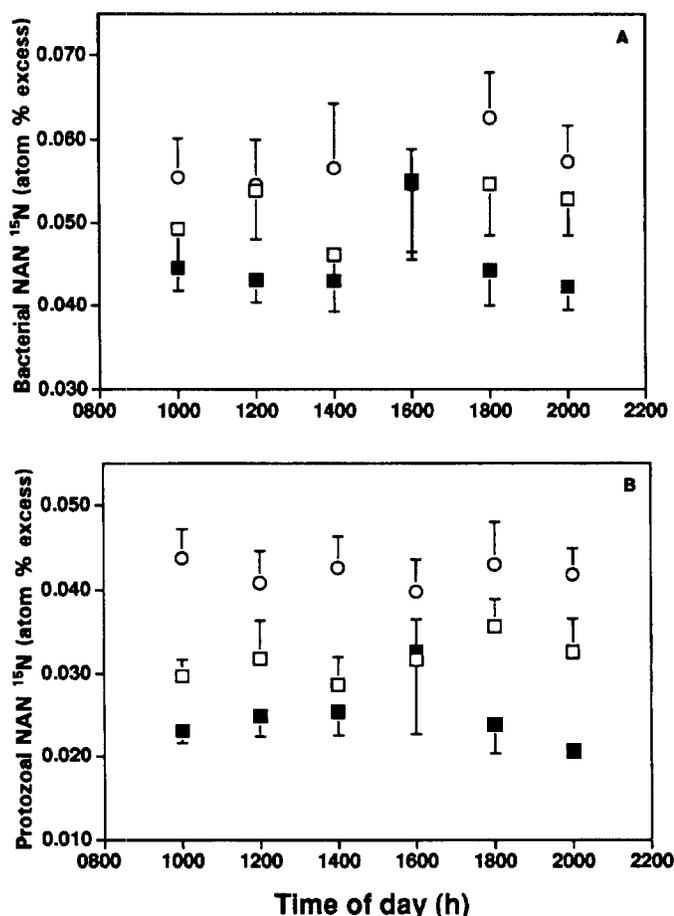


Figure 1. Enrichment of NAN ^{15}N in bacteria (a) and protozoa (b) isolated from the liquid phase over a 10-h period, 1000 to 2000 h (48 to 58 h after starting $^{15}\text{NH}_3$ infusion) on d 4, for cows fed alfalfa silage (■), alfalfa hay (□), or corn silage plus urea (○). Vertical bars represent one standard deviation.

diets consumed different amounts of DM, OM, total N, NAN, and NE_L (Table 2). The DMI of cows fed the CS diet was about 85 and 95% of that for cows fed the AS and AH diets, respectively. Lower DMI and CP and NAN contents of CS resulted in considerably lower intakes of total N and NAN for cows fed that diet. Addition of 2.2% urea (DM basis) to CS resulted in an NPN content, as a proportion of total N, that was greater than that of the other two diets (Table 1). Digestibilities of DM, OM, and NDF, determined using indigestible ADF as internal marker (8), were higher for the CS diet than for the two alfalfa diets. Similarly, digestibility of CP was 12 percentage units higher for cows fed the CS diet despite its lower CP. Ruminally digested OM was computed by assuming that 76% of apparent total tract digestion occurred in the rumen (18). Cows fed CS tended ($P = 0.08$) to

obtain more ruminally digested OM than did those fed AH.

Nolan and Leng (29) stated that continuous, intraruminal infusion of $^{15}\text{NH}_3$ was the best approach to determine the proportion of microbial NAN in total ruminal NAN. A prerequisite for reliable estimation of microbial NAN is achieving steady-state labeling of microbial protein with ^{15}N . Pilgrim et al. (33) reported that ^{15}N enrichment of ruminal microorganisms in sheep reached steady-state conditions by 78 h of infusion in one trial and by 48 h in a second trial. Petri and Pfeffer (32) found that ^{15}N enrichment of bacterial NAN in goats reached a plateau by 48 h of infusion. Firkins et al. (13) reported that, for cattle, ^{15}N enrichment of duodenal NAN attained 0.400 (48 h) and 0.407 (85 h) atom % excess during continuous infusion of $^{15}\text{NH}_3$, suggesting the error in estimation of bacterial protein flow with a 48-h infusion would be <2%. In our study, 48 h were allowed for steady-state ^{15}N labeling of both fluid and particle-associated ruminal microbes before repeated sampling for 10 h. Over this period (from 48 to 58 h after starting $^{15}\text{NH}_3$ infusion), ^{15}N enrichment of bacterial or protozoal NAN in the LP was not increased (Figure 1); the effect of time was not significant for ^{15}N enrichment of either bacterial ($P = 0.29$) or protozoal ($P = 0.59$) NAN. Mean NAN enrichments over the 10-h period were used in further computations.

As expected, the highest ^{15}N enrichments and proportions of microbial NAN were associated with LP; 63% (AS and AH) and 86% (CS) of the total NAN were estimated to be of microbial origin (Table 3). The proportion was lower for SP, ranging from 40% (AH) to 68% (CS). Microbial NAN in LP and SP NAN might have been greater for cows fed CS because NAN consumption for cows fed CS was only 30% of that for cows fed AS and 38% of that for cows fed AH (Table 2). Proportions of microbial NAN derived from NH_3 were estimated (17) by dividing the 10-h mean ^{15}N enrichment of the bacterial pools and protozoal NAN pools (Table 3) by $^{15}\text{NH}_3$ enrichment at 72 h (just before stopping ^{15}N infusion). Mean estimates were 57% (AS), 46% (AH), and 82% (CS) for bacterial NAN and 32% (AS), 28% (AH), and 62% (CS) for protozoal NAN; variation was substantial ($\text{SE} = 7.7\%$ for bacterial NAN and 5.8% for protozoal NAN). Values for the alfalfa forages were lower than the 80% (28) and 62 to 78% (36) reported for bacteria and the 35 to 64% (36) reported for protozoa. However, others observed comparable proportions: 50 to 65% for bacteria (21), 42% for mixed ruminal microbes (1), and 31 to 55% for protozoa (1). When diets containing 0.4 to 1.2% urea were fed to cattle, Firkins et al. (13) found that a mean 71% of bacterial NAN and 51% of protozoal NAN were derived from ruminal NH_3 . Proportions

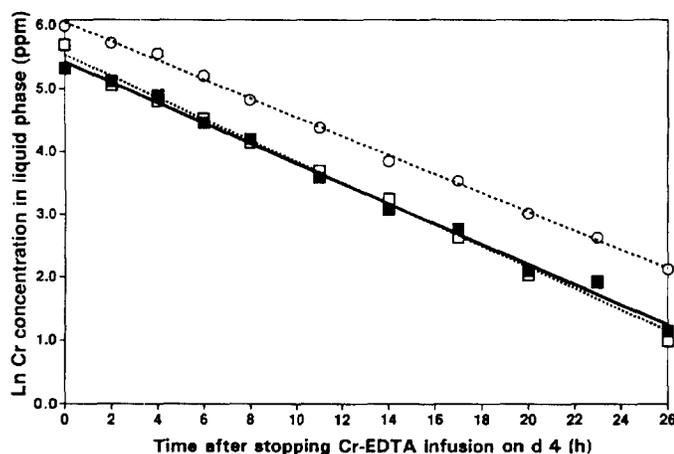


Figure 2. Plots of ln Cr concentration in the ruminal liquid phase versus time after stopping continuous infusion of Cr-EDTA at 1000 h on d 4 for cows fed alfalfa silage (AS) (■), alfalfa hay (AH) (□), or corn silage (plus urea) (CS) (○). $Y_{AS} = -0.161$ (SE = 0.004) $X + 5.491$ (SE = 0.058); $r^2 = 0.995$. $Y_{AH} = -0.169$ (SE = 0.005) $X + 5.544$ (SE = 0.075); $r^2 = 0.992$. $Y_{CS} = -0.150$ (SE = 0.002) $X + 6.057$ (SE = 0.032); $r^2 = 0.998$.

were similar for the CS plus urea diet. Lower NAN and greater NPN contents in the CS plus urea diet likely gave rise to greater protozoal ^{15}N enrichments because protozoa utilized relatively more ^{15}N enriched bacterial NAN and relatively less unenriched feed NAN for their own protein synthesis.

Cows fed the CS diet had less wet whole ruminal contents, as well as drier contents, lower percentage of DM, and less NAN in LP and SP (Table 4). However, amounts of digesta in SP, both on wet and dry bases, did not differ among diets. The LP accounted for 10.3, 10.5, and 9.9% of total ruminal DM for diets AS, AH, and CS, respectively. The concentration of NAN in the DM and the size of the NAN pool in both LP and SP also were lower for the CS diet (Table 4). The LP contained 17, 18, and 22% of the total ruminal NAN for AS, AH, and CS, respectively. The content of microbial NAN of the LP was higher for cows fed CS (Table 5), probably because of the lower NAN intake and the more favorable ratio of energy to CP on this diet. The smallest microbial NAN pool in SP was associated with the AH diet. Feed NAN content and flow, as well as pool sizes of total NAN in LP and SP, all were lower for cows fed the CS diet (Table 5). Flow of microbial NAN with LP was not different among diets, averaging 120 g/d. Flow of microbial NAN with LP represented slightly more than half of the total flow of microbial NAN from the rumen. Because of its low NAN content, passage of feed NAN for cows fed the CS diet averaged only 35% of that for cows fed the AS and AH diets.

Passage rate of LP, estimated from ruminal disappearance of Cr-EDTA (Figure 2), did not differ among diets, averaging 0.160/h (Table 5). Broderick (4) reported a mean passage rate of 0.165/h for LP and no differences for forage source when AS, AH, or CS was fed as 60% of the DM to lactating dairy cows. Passage rates of LP in the rumen in trials with dairy cows have varied considerably. For cows fed 50% concentrate diets, LP passage rates, measured using Cr-EDTA, were 0.07 to 0.08/h for nonlactating cows (37) and 0.10 to 0.11/h for lactating cows yielding 28 kg/d of milk and eating 20 kg/d of DM (19). The LP passage rates measured using polyethylene glycol increased from 0.12 to 0.14/h when feeding frequency was increased from one to four times per day for cows fed 20 kg/d of DM of a diet containing 47% concentrate and 53% CS (38).

Slopes for the ruminal disappearance of Yb adsorbed onto forage DM were used to estimate passage rates of SP (Figure 3). These rates ranged from 0.047/h (AH) to 0.054/h (AS) and did not differ among diets (Table 5). Similar passage rates of SP have been reported by others (37, 38). Although the amount of microbial NAN in the SP pool averaged three times that in LP, because of the more rapid passage rate for LP, flow of microbial NAN from the rumen was similar in SP and LP (Table 5). On average, 2.4 times more feed NAN passed from the rumen with SP than with LP; SP accounted for 70% of outflow of feed NAN. Our results indicated that 39% (AS), 44% (AH), and 43% (CS, subtracting the urea N) of the NAN that was consumed in the diet

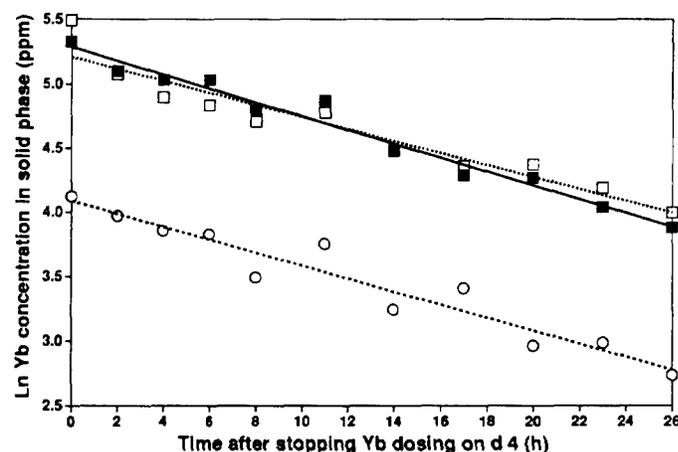


Figure 3. Plots of ln Yb concentration in the ruminal solid phase versus time after stopping Yb dosing at 1000 h on d 4 for cows fed alfalfa silage (AS) (■), alfalfa hay (AH) (□), or corn silage (plus urea) (CS) (○). $Y_{AS} = -0.054$ (SE = 0.003) $X + 5.286$ (SE = 0.044); $r^2 = 0.973$. $Y_{AH} = -0.047$ (SE = 0.005) $X + 5.209$ (SE = 0.068); $r^2 = 0.917$. $Y_{CS} = -0.050$ (SE = 0.005) $X + 4.088$ (SE = 0.070); $r^2 = 0.925$.

escaped the rumen undegraded. These estimates were greater than the ruminal protein escape percentages reported by the NRC (26): 23% (AS), 28% (AH), and 31% (CS).

When AH was fed, total flow of microbial NAN from the rumen (212 g/d) was lower than that for cows fed AS (235 g/d) or CS (243 g/d) (Table 5). The ^{15}N enrichment of bacterial NAN alone was used to compute flows of microbial NAN from the rumen; ^{15}N enrichment of isolated protozoal NAN averaged only 54% (AS), 61% (AH), and 75% (CS) of bacterial NAN (Table 3). If protozoal NAN represented 25% of the total outflow of microbial NAN (20), then dilution by the lower protozoal ^{15}N enrichments would have resulted in the underestimation of flow of microbial NAN by 11% (AS), 10% (AH), and 6% (CS). Yield of microbial NAN, computed from mean NE_L intake (26), was 249 g/d; our observed mean for flow of microbial NAN was 230 g/d. Assuming that 76% of the apparent total tract digestion of OM occurred within the rumen on all three diets (18), estimated efficiencies of microbial protein synthesis for AS, AH, and CS were, respectively, 32, 33, and 33 g of NAN/kg of OM apparently digested in the rumen. These efficiencies fell midrange among those summa-

rized by Oldham (31). However, total tract digestibilities of OM were estimated using an internal marker (indigestible ADF) and assuming the same ruminal OM digestibility (76% of total tract) for all three diets. Nelson and Satter (27) observed greater total tract digestibilities of DM and OM for cattle fed diets similar to those fed in the present study, suggesting that these digestibilities might have been underestimated by the indigestible ADF method. If so, efficiency of microbial protein synthesis would have been overestimated in this study.

Total flow of NAN (Table 5) represented 74, 85, and 89% of total N intake for the AS, AH, and CS plus urea diets, respectively, suggesting relatively better utilization of CP on AH and CS diets than on AS diets. Based on NAN flows that were estimated from sampling duodenal digesta, Merchen and Satter (22) reported that between 68 and 75% of total N intake passed the duodenum as NAN on three AS diets and one AH diet.

Rate of outflow of microbial NAN from the rumen also was estimated from regression of $\ln^{15}\text{N}$ enrichment of bacterial NAN on time (Figure 4). These rates averaged only 58% of the passage rates of Cr-EDTA but were 24% more rapid than the passage

TABLE 5. Pools of microbial and feed NAN and flow rates in liquid and solid phases of the rumen.^{1,2}

Item	AS	AH	CS	SE	<i>P</i> > <i>F</i> ³
Liquid phase					
Total NAN, g	47.6 ^a	47.3 ^a	40.8 ^b	3.3	0.011
Microbial NAN, % total N	63.1 ^b	63.3 ^b	86.3 ^a	2.1	<0.001
Microbial NAN, g	30.2 ^b	29.9 ^b	35.1 ^a	2.3	0.006
Feed NAN, g	17.4 ^a	17.5 ^a	5.7 ^b	1.1	<0.001
\ln Cr-EDTA slope, /h	0.161	0.169	0.150	0.015	0.688
Microbial NAN, ⁴ g/d	118.1	119.1	122.3	7.8	0.732
Feed NAN, ⁵ g/d	67.9 ^a	69.5 ^a	19.0 ^b	3.4	<0.001
Solid phase					
Total NAN, g	229.9 ^a	212.4 ^a	147.3 ^b	9.9	<0.001
Microbial NAN, % total N	40.9 ^b	39.8 ^b	67.9 ^a	1.4	<0.001
Microbial NAN, g	93.4 ^a	84.2 ^b	98.4 ^a	4.2	<0.001
Feed NAN, g	136.5 ^a	128.2 ^a	48.9 ^b	6.4	<0.001
\ln Yb slope, /h	0.054	0.047	0.050	0.001	0.082
Microbial NAN, ⁴ g/d	116.8 ^a	92.9 ^b	120.1 ^a	5.4	<0.001
Feed NAN, ⁵ g/d	169.4 ^a	141.8 ^b	59.3 ^c	6.3	<0.001
Total flow					
Microbial NAN, ⁶ g/d	235.0 ^a	212.2 ^b	242.5 ^a	10.4	0.002
Feed NAN, ⁷ g/d	237.3 ^a	211.5 ^b	78.3 ^c	6.4	<0.001
Total NAN, g/d	472.3 ^a	423.7 ^b	320.7 ^c	16.3	<0.001

^{a,b,c}Means within the same row without a common superscript differ ($P < 0.05$).

¹AS = Alfalfa silage, AH = alfalfa hay, and CS = corn silage.

²Means from ruminal dumping; measurements were taken at 0 and 3 h after feeding.

³Probability of a dietary effect.

⁴Flow of liquid or solid microbial NAN (grams per day) = microbial NAN in liquid or solid phase \times Cr-EDTA or Yb dilution rate \times 24.

⁵Flow of liquid or solid feed NAN (grams per day) = feed NAN in liquid or solid phase \times Cr-EDTA or Yb dilution rate \times 24.

⁶Flow of total microbial NAN (grams per day) = flow of microbial NAN in liquid phase + flow of microbial NAN in solid phase.

⁷Flow of total feed NAN (grams per day) = flow of feed NAN in liquid phase + flow of feed NAN in solid phase.

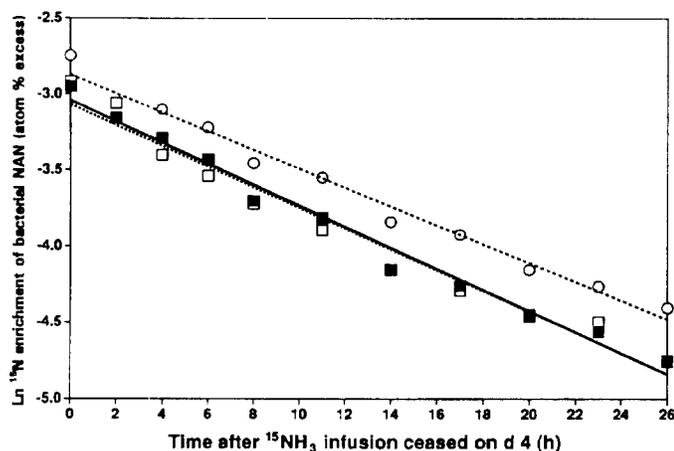


Figure 4. Plots of $\ln^{15}\text{N}$ enrichment of ruminal bacterial NAN versus time after stopping continuous infusion of $^{15}\text{NH}_3$ at 1000 h on d 4 for cows fed alfalfa silage (AS) (\blacksquare), alfalfa hay (AH) (\square), or corn silage (plus urea) (CS) (\circ). $Y_{AS} = -0.069$ (SE = 0.003) $X - 3.039$ (SE = 0.042); $r^2 = 0.985$. $Y_{AH} = -0.068$ (SE = 0.004) $X - 3.069$ (SE = 0.060); $r^2 = 0.968$. $Y_{CS} = -0.062$ (SE = 0.003) $X - 2.872$ (SE = 0.038); $r^2 = 0.984$.

rates of Yb adsorbed to feed DM (Table 5). Overall estimates of the flow of total microbial NAN based on dilution of this single bacterial ^{15}N pool were about 15% lower than those obtained by the combined Cr and Yb technique. Because of ^{15}N recycling within the rumen (28), rate of dilution of ^{15}N in bacterial protein is not as rapid as the actual rate of passage of microbial NAN out of the rumen. Intraruminal recycling of microbial N may be as high as 50% (20). Our samples represented both LP and SP bacteria, but probably were relatively rich in LP bacteria. The LP and SP bacteria have different turnover rates (11). If 35% of the bacteria associated with the SP was assumed to be recovered by our extraction procedure (11), then the turnover rate for ^{15}N bacterial NAN, based on ruminal dilution rates of Cr (LP) and Yb (SP) markers, should have averaged 0.121/h for these diets. The mean turnover rate for ^{15}N bacterial NAN, however, was 0.067/h, suggesting that about 45% of bacterial ^{15}N might have been recycled in the rumen. Thus, the dilution rate of the ^{15}N in bacterial NAN appeared to be unsatisfactory for quantifying microbial NAN production in the rumen.

After the ^{15}N infusion was stopped, ^{15}N enrichment of NH_3 decreased sharply, but the decline in ^{15}N enrichment of bacterial NAN was much slower (Figure 5). Petri and Pfeffer (32) reported similar results. However, after 2 h, ^{15}N enrichment of bacterial NAN followed the pattern of the NH_3 ^{15}N pool, despite the large variations in NH_3 concentrations,

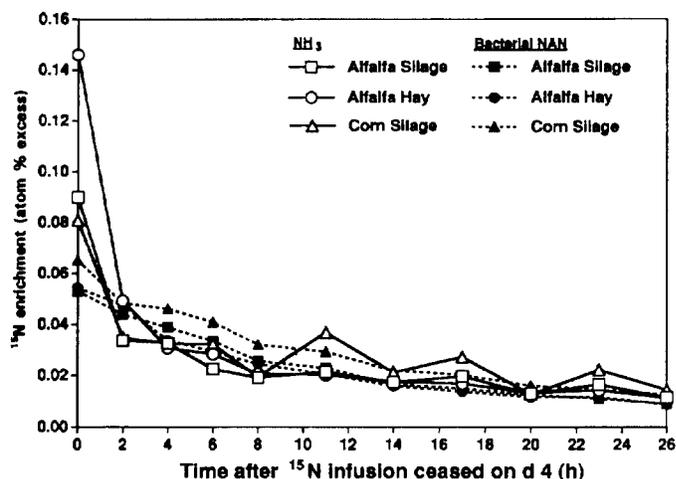


Figure 5. Changes in ^{15}N enrichment of ruminal NH_3 N and bacterial NAN after stopping continuous infusion of $^{15}\text{NH}_3$ for cows fed alfalfa silage, alfalfa hay, or corn silage (plus urea). Pooled standard errors were 0.0032 (NH_3 enrichment) and 0.0009 (bacterial NAN enrichment).

which were dependent on time after feeding (Figure 6). Although dilution of the Cr-EDTA was assumed to be representative of LP, reliability of Yb as an SP marker has been questioned because of reports of marker migration (10). The labeling method used in the present study should have slowed the rate of Yb migration for at least three reasons: 1) samples were washed to remove water-soluble DM from the forages, 2) AH samples were sieved to remove small particles that might have flowed with the LP, and 3) labeled forage samples were soaked in slightly acidic solution and then washed to remove loosely bound Yb.

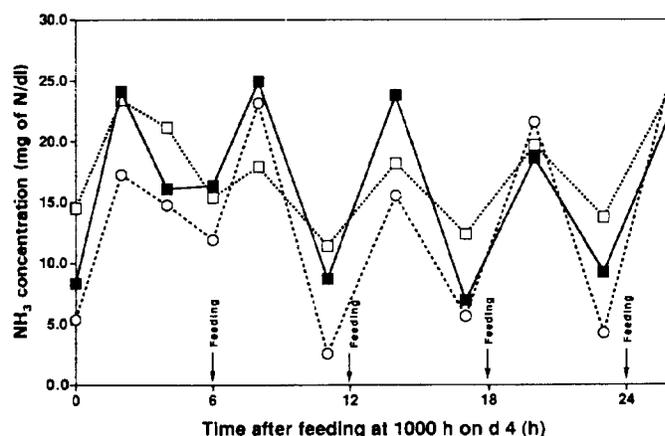


Figure 6. Concentration of NH_3 in ruminal fluid as influenced by feeding alfalfa silage (\blacksquare), alfalfa hay (\square), or corn silage (plus urea) (\circ) beginning at 1000 h on d 4; the pooled standard error was 0.46.

TABLE 6. Concentrations of N metabolites and VFA in ruminal fluid and of glucose and urea in blood plasma.¹

Item	AS	AH	CS	SE	<i>P</i> > <i>F</i> ²
Ruminal fluid					
pH	6.65 ^a	6.44 ^b	6.48 ^b	0.02	<0.001
NH ₃ , mM	11.7 ^a	12.5 ^a	9.6 ^b	0.5	<0.001
Total AA, mM	0.44 ^b	0.28 ^b	0.77 ^a	0.06	<0.001
Total VFA, mM	117.7 ^b	127.0 ^a	106.6 ^c	1.4	<0.001
VFA, mol/100 mol					
Acetate (A)	69.7 ^b	72.2 ^a	66.1 ^c	0.2	<0.001
Propionate (P)	16.9 ^b	16.3 ^c	18.6 ^a	0.1	<0.001
Isobutyrate	1.30 ^a	1.03 ^b	0.72 ^c	0.02	<0.001
Butyrate	8.5 ^b	7.7 ^c	11.2 ^a	0.1	<0.001
Isovalerate + 2-methylbutyrate	1.67 ^a	1.26 ^b	1.24 ^b	0.02	<0.001
Valerate	1.94 ^b	1.44 ^c	2.13 ^a	0.03	<0.001
A:P	4.17 ^b	4.44 ^a	3.63 ^c	0.04	<0.001
Blood plasma					
Glucose, mg/dl	86.0	85.1	87.4	2.4	0.780
Urea, mM	8.27 ^a	8.21 ^a	5.85 ^b	0.21	<0.001

^{a,b,c}Means within the same row without a common superscript differ ($P < 0.05$).

¹AS = Alfalfa silage, AH = alfalfa hay, and CS = corn silage.

²Probability of a dietary effect.

Effects of diet on concentrations of several metabolites in ruminal fluid and blood were significant (Table 6). Total VFA concentration, molar proportion of acetate, and the ratio of acetate to propionate were highest in ruminal fluid from cows fed AH. Total VFA concentration, molar proportion of acetate, and the ratio of acetate to propionate were lowest, and molar proportions of propionate and butyrate were highest, for cows fed CS. Although ruminal fluid pH was greatest for cows fed AS, that diet gave rise to intermediate concentrations of total VFA; molar proportions of acetate, propionate, and butyrate; and ratios of acetate to propionate. The concentration of total VFA tended to increase after feeding and then declined (Figure 7), although the magnitude of

change was smaller than that for ruminal NH₃ (Figure 6). Interaction of diet and time after feeding for total VFA was not significant ($P = 0.96$). As expected, ruminal NH₃ and blood urea were higher for cows fed the higher CP diets containing AS and AH. However, total AA concentrations in the rumen were greater for cows fed CS. Molar proportions of the branched-chain VFA isobutyrate and 2-methylbutyrate plus isovalerate, which are formed from microbial catabolism of branched-chain AA, generally were greater on the alfalfa diets for which degradation of dietary NAN was greater. The reason that ruminal valerate, which is formed partly from microbial breakdown of Pro (16), was greatest for the CS diet and least for the AH diet, is unknown. Blood glucose was unaffected by diet.

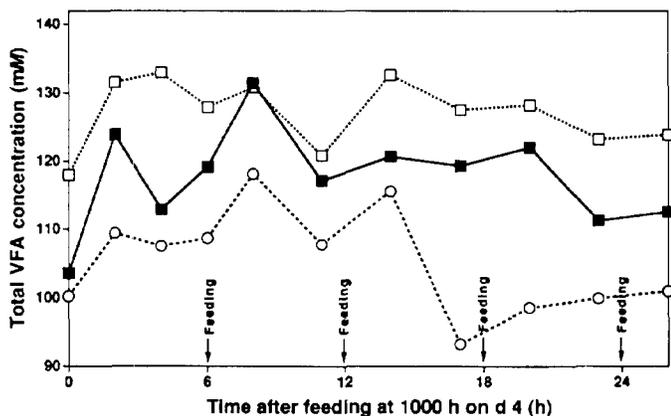


Figure 7. Concentration of total VFA in ruminal fluid as influenced by feeding alfalfa silage (■), alfalfa hay (□), or corn silage (plus urea) (○) beginning at 1000 h on d 4; the pooled standard error was 1.4.

CONCLUSIONS

Enrichment patterns after continuous infusion of ¹⁵NH₃ indicated that microbial NAN constituted 63 to 86% of total NAN in the LP and 40 to 68% of total NAN in the SP; proportions were similar for cows fed AS and AH and greater for cows fed CS plus urea. Flow of microbial NAN out of the rumen was highest for cows fed CS (243 g/d) and was lowest for cows fed AH (212 g/d), representing 50% (AS and AH) and 76% (CS) of the total flow of NAN. Flows of microbial NAN in LP and SP were about equal, despite microbial NAN in LP, averaging only 34% of that in SP. Although ruminal pools of feed NAN averaged 14 g (LP) and 105 g (SP), 30% of feed NAN left the rumen with the LP, suggesting that soluble proteins escaped to a disproportionate extent. An estimated 39% (AS), 44% (AH), and 43% (CS excluding urea

N) of feed NAN escaped the rumen undegraded. Compared with AS and AH, cows fed CS had lower flows of total NAN, probably because of lower NAN intake. Dual LP (Cr-EDTA) and SP (Yb) markers, when used with $^{15}\text{NH}_3$ to label microbial protein, yielded satisfactory estimates of passage of microbial and feed NAN in cows equipped only with ruminal cannulas.

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