Ruminal metabolism of ammonia N and rapeseed meal soluble N fraction

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ABSTRACT

The present study was conducted to investigate ruminal N metabolism in dairy cows using ^{15}N labeled N sources [ammonia N (AN), soluble non-ammonia N (SNAN) from rapeseed meal, and insoluble non-ammonia N (NAN) from rapeseed meal]. To describe the observed pattern of ^{15}N transactions in the rumen, dynamic compartmental models were developed. The experiment consisted of 3 experimental treatments allocated to 4 cows according to a changeover design. The results from 2 treatments (AN and rapeseed meal SNAN) are reported in this paper. Ammonia N and rapeseed SNAN, both labeled with ^{15}N, were administered intraruminally. Rumen evacuations in combination with grab samples from the rumen contents were used to determine ruminal N pool sizes. The ^{15}N-atom% excess was determined in N fractions of rumen digesta samples that were distributed between 0 and 82 h after dosing. For the AN treatment, a 2-compartment model was developed to describe the observed pattern in ^{15}N-atom% excess pool sizes of AN and bacterial N and to estimate kinetic parameters of ruminal ^{15}N transactions. For the SNAN treatment, an additional compartment of SNAN was included in the model. Model simulations were used to estimate N fluxes in the rumen. Both models described the observed pattern of ^{15}N-atom% excess pool sizes accurately, based on small residuals between observed and predicted values. Immediate increases in ^{15}N-atom% excess of bacterial N with AN treatment suggested that microbes absorbed AN from extracellular pools rapidly to maintain sufficient intracellular concentrations. Proportionally 0.69 of the AN dose was recovered as NAN flow from the rumen. A rapid disappearance of labeled SNAN from rumen fluid and appearance in bacterial N pool indicated that, proportionally, 0.56 of SNAN was immediately either adsorbed to bacterial cell surfaces or taken up to intracellular pools. Immediate uptake of labeled SNAN was greater than that of AN (proportionally 0.56 vs. 0.16 of the dose). Degradation rate of SNAN to AN was relatively slow (0.46/h), but only 0.08 of the SNAN dose was estimated to escape ruminal degradation because of rapid uptake by the bacteria. Overall, losses of the ^{15}N dose as AN absorption and outflow from the rumen were higher ($P < 0.01$) for the AN than the SNAN treatment (0.31 and 0.11 of the dose, respectively). Consequently, recovery as NAN flow was greater for SNAN than for AN treatment (0.89 vs. 0.69 of the dose). Estimated rate of bacterial N recycling to AN was on average 0.006/h, which suggests that N losses due to intraruminal recycling are small in dairy cows fed at high intake levels. We conclude that SNAN isolated from rapeseed meal had better ruminal N utilization efficiency than AN, as indicated by smaller ruminal N losses as AN (0.11 vs. 0.31 of the dose) and greater bacterial N flow (0.81 vs. 0.69 of the dose). Furthermore, the current findings indicate that rapid adsorption of soluble proteins to bacterial cells plays an important role in ruminal N metabolism.
Key words: rapeseed, nitrogen, ^{15}N, soluble protein, ammonia

INTRODUCTION

The role of dairy cows in human food systems has traditionally relied on efficient utilization of fibrous feed resources that cannot be utilized by monogastric animals. Another advantage that favors ruminants over monogastrics is microbial protein synthesis from NPN. Efficient utilization of nutrients contained in various feed resources requires nutritional models that account for the dynamic interactions between plant structures, rumen microbes, and the host animal. In the current protein evaluation systems for dairy cows, protein requirements have been described in terms of RDP and RUP (NRC, 2001). According to this model, microbial protein synthesis is determined by the availability of fermentable energy and RDP. Within this frame, when RDP supply is sufficient to sustain optimal microbial N synthesis, positive milk yield responses to changes

in diet composition can be achieved by increasing both energy and RUP intakes. However, manipulations of diet composition according to these principles have often failed to support the validity of the current theoretical concepts (e.g., Rinne et al., 1998; Olmos Colmenero and Broderick, 2006; Borucki Castro et al., 2008). Quite the contrary, we find increasing evidence indicating that the distinction between RDP and RUP represents an overly simplistic model of the complex rumen N metabolism. Soluble feed protein is regarded as RDP, although peptides originating from partial ruminal degradation of feed protein may comprise between 0.05 and 0.10 of total dietary NAN entering the omasal canal (Choi et al., 2002). Regardless of several studies that have demonstrated the beneficial effects of preformed amino acids on the efficiency of microbial protein synthesis (Rooke and Armstrong, 1989; Chikunya et al., 1996; Brito et al., 2007) no distinction is made between RDP sources from NPN or true protein in the current systems (NRC, 2001). Previous evidence indicates that soluble NAN (**SNAN**) supplied by well-conserved forages (mainly AA and peptides) is of higher value for ruminants than ammonia N (Ahvenjärvi et al., 2018). It is evident that further studies are needed to fully comprehend the essential features of metabolic and dynamic pathways associated with ruminal NPN and protein metabolism. Therefore, a better representation of rumen N metabolism that accounts for the multiple steps involved with protein degradation to peptides and amino acids with subsequent effects on passage dynamics should be incorporated into protein evaluation systems to encourage optimal use of feed resources and, at the same time, to ensure a balanced nutrient supply to the animal.

The objectives of the present study were to investigate the ruminal metabolism of ammonia N and protein fractions isolated from rapeseed meal and to develop models that represent ruminal metabolism of these N fractions. Our hypotheses were that NAN, both soluble and insoluble, is used more efficiently for microbial synthesis than ammonia N (**AN**), and that part of

NAN escapes microbial metabolism in the rumen. To evaluate these hypotheses, 3 treatments were assigned to cannulated lactating dairy cows: ^{15}N-labeled AN, and ^{15}N-labeled rapeseed meal fractionated to SNAN and insoluble NAN. The data on ruminal metabolism of insoluble NAN was excluded from this report, because full descriptions of that data and modifications to the current model would have extended the paper considerably.

MATERIALS AND METHODS

Experimental Design, Animals, and Diet

A changeover experiment with 4 Nordic Red dairy cows and three 14-d experimental periods was conducted to evaluate the ruminal metabolism of 3 different N fractions. The treatments used in this study were ^{15}N-labeled AN, and SNAN and insoluble NAN fractions of ^{15}N-labeled rapeseed meal (**RSM**). The allocation of AN, SNAN, and insoluble NAN treatments to experimental animals is shown in Table 1. The data presented in this report were obtained from the AN and SNAN treatments.

The cows were fitted with a rumen cannula (Bar Diamond, Inc., Parma, ID). Cows produced (mean ± SD) 32.9 ± 1.42 kg of ECM, and were of 3 ± 1.3 parity, 155 ± 59.2 DIM, and 614 ± 21.7 kg BW at the beginning of the experiment. The ECM was calculated according to Sjaunja et al. (1991). Cows were housed in individual tiestalls in a dedicated metabolism unit with free access to water and salt blocks. They were milked twice daily at 0700 and 1700 h. Daily DM and N intakes were on average 22.4 kg/d (SD 0.88) and 557 g/d (SD 21.7), respectively. The Animal Experiment Board in Finland (Hämeenlinna, Finland) approved all experimental procedures in accordance with the guidelines established by the European Community Council Directive 86/609/EEC.

Cows were offered TMR that consisted of grass silage and concentrates, with forage-to-concentrate ratio of 60:40 on a DM basis. The ration was fed in 4 equal meals at 0600, 0900, 1800, and 2000 h, with 5 to 10% allowance for refusals. Silage was prepared from a primary growth of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) sward ensiled with a formic acid–based additive (AIV 2 Plus, Kemira Ltd., Helsinki, Finland) applied at a rate of 4.9 L/t. The concentrate consisted of (g/kg of DM) rolled barley (243), rolled oats (243), molassed sugar beet pulp (243), solvent-extracted RSM (243), and a proprietary vitamin and mineral premix (28; Onni-Kivennäinen, Melica Ltd., Paimio, Finland). Chemical compositions of experimental feeds and TMR are shown in Table 2.

Table 1. Allocation of ^{15}N-labeled treatments to experimental animals[1]

| Cow | Experimental period | | |
	1	2	3
1	SNAN	AN	INAN
2	SNAN	AN	INAN
3	INAN	AN	SNAN
4	INAN	AN	SNAN

[1]Treatments comprised ruminal administration of ^{15}N-labeled ammonium sulfate (AN), soluble fraction of rapeseed meal (SNAN), and insoluble fraction of rapeseed meal (INAN).

Table 2. Chemical composition (g/kg of DM) of grass silage, concentrates, and ^{15}N-labeled rapeseed meal

Item	Grass silage[1]	Concentrate	TMR	^{15}N-labeled rapeseed meal
DM, g/kg	243	944	346	939
In DM, g/kg				
Ash	63	79	69	87
N	21.4	30.2	24.9	61.5
NDF	536	258	425	267
iNDF[2]	58	72	64	—
pdNDF,[3] g/kg of NDF	892	721	824	—

[1]Fermentation quality: pH 3.76. In DM (g/kg): lactic acid (54), acetic acid (23), propionic acid (0.2), butyric acid (0.1), ethanol (10), reducing sugars (30). In total N (g/kg): ammonia N (35).

[2]iNDF = indigestible NDF, determined based on 12-d ruminal in situ incubation.

[3]pdNDF = potentially digestible NDF, determined as NDF − iNDF.

Labeled N Sources

Ammonium sulfate $[(NH_4)_2SO_4]$ with 10% of ^{15}N/N (Isotec, Miamisburg, OH) was used as a source of labeled AN. Labeled SNAN was prepared from ^{15}N-enriched rapeseed (*Brassica rapa* L. ssp. oleifera DC, variety Valo, Boreal Plant Breeding Ltd., Jokioinen, Finland) grown in 3 experimental plots (1.25 m × 8.00 m) established on a field in Jokioinen, Finland (60°49′N, 23°28′E). At sowing, compound fertilizer was applied at a rate of 6.6 kg/ha of N, 26.7 kg/ha of P, and 30.8 kg/ha of K. Once cotyledons had emerged, 1,132 g of labeled ammonium sulfate (240 g of N; 80 kg/ha of N) was dissolved in 218 L of water and manually applied on experimental plots using a 10-L watering can. In brief, whole rapeseeds were ground and oil was removed by repeated extraction using diethyl ether. Extracted RSM was allowed to stand overnight at room temperature, then dried at 60°C for 24 h, and homogenized using a mortar and pestle. A 100-g sample was collected for chemical analysis, milled through a 1-mm screen, and analyzed for DM, ash, total N, NDF, N fractions (Cornell Net Carbohydrate and Protein System; Higgs et al., 2015), AA, and ^{15}N/N enrichment of CNCPS fractions. The remainder of ^{15}N-labeled RSM was split into 4 equal portions of 805 g of DM and stored at −20°C until fractionated to SNAN and insoluble N as described herein.

Extraction of Soluble N

One day before ruminal administration during experimental periods 1 and 3, RSM was thawed at room temperature and separated into soluble and insoluble N-enriched fractions. The SNAN fraction of each 805-g (DM) batch of RSM was extracted using 2.5 L of bicarbonate-phosphate buffer (0.010 M Na_2HPO_4, 0.011 M KH_2PO_4, 0.117 M $NaHCO_3$). Following the addition of buffer, the mixture was allowed to stand for 2 h at room temperature with occasional stirring. Thereafter, it was squeezed through a 38-μm polyester filter fabric to remove buffer with soluble components. The insoluble residue was rinsed twice with 2 L of buffer and filtered. Small particles suspended in the soluble fraction were removed by centrifugation at 10,000 × g for 15 min at 4°C. The supernatant was removed by aspiration, and four 50-mL samples were collected and frozen immediately at −20°C before determination of total N and PA_2-fraction (soluble true protein in the updated CNCPS; Van Amburgh et al., 2015) concentrations, and ^{15}N enrichment. Soluble N was stored at 4°C overnight before mixing with rumen contents.

Administration of Labeled N and LiCoEDTA into the Rumen

Details of administration of labeled N and LiCoEDTA into the rumen have been reported previously (Ahvenjärvi et al., 2018). In brief, the experimental animals were assigned to 1 of 2 groups. Treatments were administered to the first pair of cows on d 9 and to the second pair on d 10 of each experimental period. Starting at 0635 h, the entire rumen contents were evacuated into two 50-L barrels, weighed, and maintained warm in a water bath. At 0655 h, rumen contents were transferred into plastic feed carts and mixed thoroughly, and three 200-g samples of rumen contents were collected for determination of rumen total N pool size, concentrations of N fractions, and background ^{15}N-atom% of each N fraction (AN, SNAN, and bacteria N). To stop the enzymatic activity, 100 g of 0.9% (wt/vol) NaCl solution with 4 g/L of $HgCl_2$ and 100 g of 0.9% (wt/vol) NaCl solution with 0.2 N H_2SO_4 were added to the samples, and the samples were mixed manually and frozen at −20°C. At 0700 h labeled N sources and LiCoEDTA (5 g dissolved in 500 mL of water) were mixed manu-

ally with rumen contents. A spot sample of 200 g was collected for the analysis of N concentration and ^{15}N-atom% excess (**APE**) of each N fraction.

Ruminal Sampling

Grab samples of rumen contents were collected during rumen evacuation (at 0 h), and at 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 14, 17, 22, 27, 33, 39, 47, 55, 63, 72, and 82 h after administration of labeled N sources. Four spot samples, each approximately 250 g, were collected from the reticulum, the ventral sac, and the anterior and posterior sites of the dorsal sac. To collect a sample from the reticulum, a 250-mL wide-necked bottle was used. Samples were composited and mixed thoroughly, a 200-g subsample was obtained, and the rest of the sample was returned into the rumen.

Difficulties in obtaining grab samples that are representative of true digesta present in the rumen have long been recognized (Faichney 1980). To avoid such problems related to the tendency of rumen solids and liquid to separate during sample collection, grab samples obtained from the rumen were separated into solid and liquid fractions with the assumption that each phase in the grab sample is representative of that in the rumen digesta. As described below, concentrations of N fractions in solids and liquid phases collected at frequent intervals from the rumen were combined with the data from rumen evacuations to calculate rumen pool sizes of AN, soluble N, and insoluble N.

Rumen Pool Sizes

Rumen pool sizes of N fractions were determined based on the quantity of digesta evacuated from the rumen and samples collected from the whole rumen contents. Details of the rumen evacuation procedures have been reported previously (Ahvenjärvi et al., 2018). Rumen evacuations were started at 0635 h on d 4, 1100 h on d 9, and 1730 h on d 12 of each period. Digesta samples were obtained at 0655 h (1 h after morning meal) on d 4, at 1130 h on d 9, and at 1800 h on d 12, for the first pair of cows. Sampling was advanced by 24 h for the second pair of cows. Samples were collected and treated as described above.

Fractionation of Rumen Digesta Samples

Rumen digesta samples were separated into solid and liquid phases and bacterial fraction. Once thawed overnight at room temperature, digesta samples were squeezed through a 38-μm polyester filter fabric to remove solubles. Solids remaining on the filter were homogenized with 400 mL of 0.9% NaCl (wt/vol) solu-tion in a blender for 1 min. The suspension was filtered through the same 38-μm polyester filter fabric. Solubles were removed by squeezing, and the second filtrate was pooled with the first one. Small particles and protozoa in pooled filtrate were recovered by centrifugation at $500 \times g$ for 7 min at 4°C. The supernatant was removed by aspiration, and the pellet was combined with the solids retained on the filter. The solid fraction, which consisted of feed particles, residual soluble matter retained with feed particles, and microbial cells, was weighed and freeze-dried before determination of total N and APE. Supernatant obtained after low-speed centrifugation was centrifuged at $10,000 \times g$ for 30 min at 4°C to sediment bacterial cells. The high-speed pellet was assumed to contain only bacterial cells, but potential contamination with feed particles was not verified. Bacterial pellets were frozen at −20°C, freeze-dried, and submitted to total N and APE determinations. The supernatant, defined as rumen liquid, was removed by aspiration, weighed, and analyzed for Co, total N, AN, and SNAN concentrations and APE of N fractions.

To determine rumen pool sizes, subsamples of rumen liquid were analyzed for total N and AN concentrations. Bacterial pellets were combined with the solids retained on the polyester filter fabric to obtain a TS fraction that was weighed, frozen at −20°C, and freeze-dried. The samples were kept in an oven overnight at 50°C to remove residual moisture. The solids were weighed, milled using a hammer mill with 1-mm screen, and analyzed for total N concentration.

Chemical Analysis

Details of chemical analysis have been reported previously (Ahvenjärvi et al., 2018). In brief, N concentrations of dry samples were determined using a Dumas-type N analyzer (Leco FP-428; Leco Corporation, St. Joseph, MI). For the analysis of APE, dry samples were homogenized using a ball mill (Mixer Mill 301, Retsch, Haan Germany). A sample equal to 100 μg of N was enclosed within a tin capsule, and APE was determined using a Hydra 20-20 isotope ratio mass spectrometer linked to an elemental analyzer (Sercon Ltd., Crewe, UK).

Amino acids were analyzed according to the method established by the European Commission (1998). Amino acids were determined by reaction with ninhydrin using a Biochrom 20 AA analyzer (Pharmacia Biotech, Cambridge, UK) equipped with a 90×4.6-mm PEEK sodium pre-wash column and 250×4.6-mm Bio PEEK sodium high-performance column (Pharmacia Biotech) after acid hydrolysis (6 M HCl, 110°C, 24 h). The sulfur-containing AA cysteine and methionine were oxidized with performic acid (0°C, 16 h) to cysteic acid

and methionine sulfone before acid hydrolysis and calculated as cystine and methionine, respectively. The hydrolysis acid contained 1 mg of phenol/mL of acid to protect labile AA, especially tyrosine and phenylalanine, both of which were determined in hydrolysates of unoxidized samples. Other AA were determined as mean values of the oxidized and unoxidized samples. Cobalt concentrations were analyzed in rumen digesta samples collected before mixing of LiCoEDTA with rumen contents (blank samples), immediately afterward, and at 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h post-administration.

Calculations

Rumen-soluble N pool size was calculated based on soluble N concentrations in rumen digesta grab samples, and rumen fresh matter pool size was determined as a mean of 3 rumen evacuations:

$$\text{Rumen-soluble N pool size (g)} =$$
$$\text{Rumen fresh matter pool size (kg)}$$
$$\times \text{ Soluble N concentration in rumen}$$
$$\text{digesta (g/kg).} \qquad [1]$$

Rumen pool size of AN was calculated similarly to Equation [1], whereas SNAN pool size was calculated as the difference between those of rumen-soluble N and AN. To calculate APE of different N fractions, the background ^{15}N-atom% was analyzed from bacteria and digesta samples collected before administration of labeled N sources into the rumen. Pool size of rumen-soluble ^{15}N in excess of background levels (^{15}**NEP**) was calculated as

$$\text{Rumen } ^{15}\text{NEP of soluble N (mg)} =$$
$$\text{Rumen-soluble N pool size (mg)}$$
$$\times \text{ APE of soluble N.} \qquad [2]$$

The APE of insoluble N was not analyzed directly but was calculated as a weighted mean of APE of bacteria N and solids N. The fractional proportions of unlabeled feed N and bacteria N in rumen-insoluble N pool size were predicted based on an assumption that APE of rumen-insoluble N pool is a weighted mean of APE of each subfraction:

$$\text{Rumen-insoluble N (APE)} = a \times \text{Feed N (APE)}$$
$$+ b \times \text{Bacteria N (APE).} \qquad [3]$$

where a and b are fractional proportions of feed N and bacterial N in rumen insoluble N pool size. The sum of a + b was constrained to 1.

All APE of the insoluble N pool was assumed to be microbial N. Estimated transfers of AN and SNAN from the reticulo-rumen, based on model simulations, were analyzed using a model $Y = \mu + A_i + P_j + T_k + \varepsilon_{ijkl}$, where μ is the overall mean, A_i represents the random effect of animal, P_j and T_k are the fixed effects of period and diet, respectively, and ε_{ijkl} represents the residual variation. The statistical analysis was carried out using the MIXED procedure of SAS (SAS Inc. 2002–2012, Release 9.4; SAS Institute Inc., Cary, NC).

Model Development

The model describing AN kinetics was similar to that used in our previous study (Ahvenjärvi et al., 2018), except that ^{15}NEP of rumen protozoa was not determined in the present study. The model consisted of 2 compartments, AN and bacterial N (Figure 1A). We fitted the model to the data using WinSAAM version 3.0.7 software (http://www.winsaam.org/; New Bolton Center, Biostatistics Unit, University of Pennsylvania; Wastney et al., 1998). The parameter estimates were iteratively adjusted until the minimum of the sum of squared differences between observed and model-predicted values was attained. The standard output in WinSAAM includes estimates for each parameter with fractional standard deviation (**FSD**) and an array of observed versus predicted data values. In the model, AN entering the rumen disappears via direct absorption through the rumen wall, liquid outflow entering the omasal canal, or synthesis to bacterial N. Because AN absorbed or flowing out in the liquid phase are both metabolized to urea in the liver, these rates were combined to represent the total disappearance rate of AN. Bacterial N synthesized from extracellular AN disappears via entering the omasal canal and recycling to AN pool. Because of instant uptake of AN to bacterial N pool, initial bacterial N pool was estimated as an adjustable parameter.

A 3-compartment model was fitted to the ^{15}NEP data for each cow given a single dose of ^{15}N-labeled SNAN (Figure 1B). According to the model, SNAN escapes the rumen via entering the omasal canal in the liquid phase and is degraded to AN or incorporated directly into bacterial N. Bacterial N disappears through entering the omasal canal or is recycled to the AN pool. To represent an immediate transfer of SNAN to bacterial N, an initial condition at 0 h was estimated for bacterial N pool. Parameter estimates derived for AN kinetics in the AN model were used in the SNAN model to

avoid over-parameterization. Observed values of ^{15}NEP for SNAN were greater than predicted after about 18 h post-dose. This could be due to the lysis of bacterial cells during sample processing, which increases the ^{15}NEP of SNAN. To account for this artifact, an adjust-

able parameter predicting the proportion of bacterial N released to the SNAN pool was estimated. Liquid passage rate determined by using CoEDTA as a marker was used to estimate escape of SNAN. Quantitative estimates of N fluxes in the rumen were derived by model simulations using Powersim version 2.5 software (Powersim AS, Isdalstø, Norway). Model simulations provided estimates for the contribution of each metabolic pathway to disappearance of 1,000 mg of AN or SNAN from the reticulo-rumen. Differential equations of the model are presented in Supplemental File S1 (https://doi.org/10.3168/jds.2019-17761).

RESULTS

Labeled N and Rumen Environment

Grass silage used in the study was of high quality in terms of both nutritive value and hygienic quality, as indicated by the low indigestible NDF concentration and low concentrations of AN and fermentation acids (Table 2). The composition of ^{15}N-labeled RSM was similar to tabulated values (NRC, 2001; Luke, 2019). The amounts of N administered as pulse doses into the rumen were 4.98 and 16.4 g for AN and SNAN treatment, respectively (Table 3). The proportion of true protein N in the SNAN fraction was 514 g/kg, the rest being mainly free AA and peptides. Total AA comprised 888 g/kg of CP of rapeseed meal SNAN.

Dry matter intake (22.2 vs. 22.7 kg/d) and ECM yield (32.7 vs. 32.9 kg/d) were similar for AN and SNAN treatments, respectively. Rumen pool sizes of total N (379 vs. 384 g), NAN (373 vs. 379 g), and ammonia N (5.6 vs. 4.8 g), for AN and SNAN treatments, respectively, were not influenced by the treatment. Bacterial N pool sizes (estimated using Eq. [3]) were, on average, 200 g (SD 26.9) and 236 g (SD 20.8) for AN and SNAN
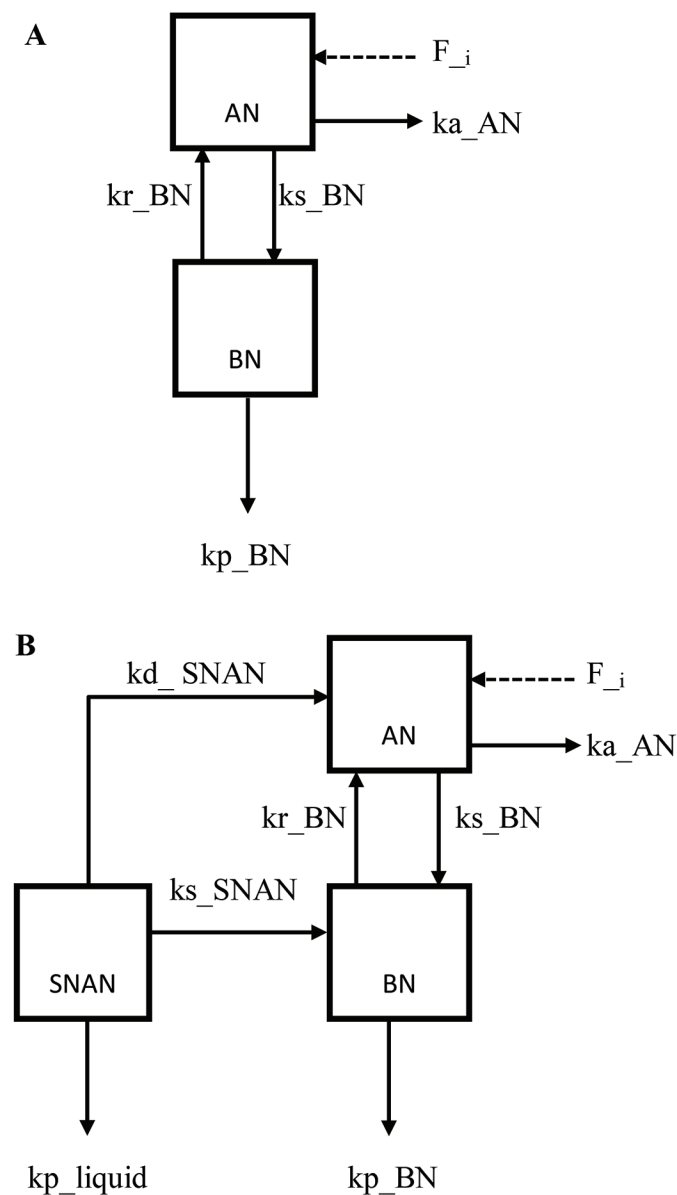

Figure 1. Schematic presentation of ammonia N (A) and soluble NAN (B) models to describe ^{15}N metabolism in the rumen. AN, SNAN, and BN represent pool sizes (mg) of ammonia N, soluble NAN, and bacterial N, respectively. F_i = external input flux of AN and urea N recycling (mg/d); ka_AN = fractional rate of AN disappearance from the rumen (absorption + passage); ks_BN = fractional rate of AN synthesis to bacterial N; ks_SNAN = fractional rate of direct uptake of SNAN by bacteria; kd_SNAN = fractional rate of degradation of SNAN to ammonia N; kr_BN = fractional rate of BN recycling to AN; kp_liquid = fractional passage rate of liquid matter from the reticulo-rumen to the omasal canal; kp_BN = fractional passage rate of BN from the reticulo-rumen to the omasal canal.

Table 3. Amount and chemical composition of ^{15}N-labeled ammonium sulfate and rapeseed meal soluble N administered into the rumen[1]

Item	Ammonium sulfate	Soluble rapeseed meal protein[1]
Fresh matter, g	23.5	5,268 (±66.4)
DM, g/kg	—	49 (±0.03)
Total N, g	4.98	16.4 (±0.01)
AA, g	—	91.1 (±0.78)
N composition, g/kg of total N		
Ammonia N	1,000	—
AA and peptide N	—	486 (±2.5)
Soluble true protein N	—	514 (±2.5)
^{15}N/N-atom% in excess		
Total N	9.62	2.18 (±0.006)
Ammonia N	9.62	—
Total ^{15}N, mg in excess[2]	479	357 (±0.8)

[1]Values (± SD) based on n = 2 determinations.

[2]Amount of ^{15}N administered in excess of background enrichment.
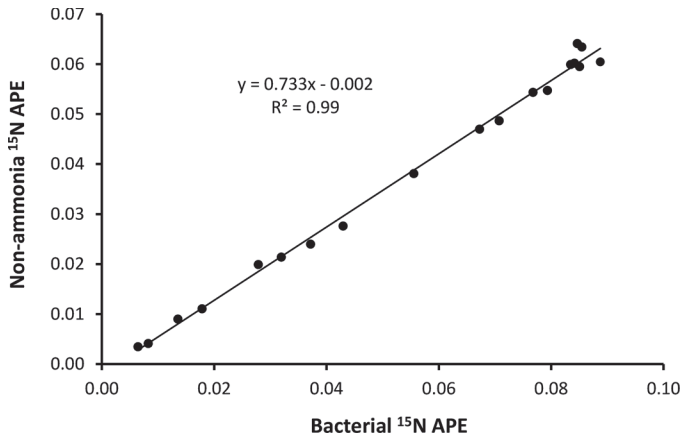
Figure 2. An example of the proportion of bacterial N in NAN predicted based on the relationship between ^{15}N-atom% excess (APE) of rumen NAN and that of bacterial N. This figure represents an example for 1 animal that received a single dose of labeled soluble NAN. The slope of regression indicates that, for this animal, 0.733 ($P < 0.01$) of rumen NAN was bacterial N.

treatments, respectively. An example of the predicted proportion of bacterial N in NAN for one animal that received a single dose of labeled SNAN is presented in Figure 2. On average, bacterial N represented 0.53 (SD 0.026) and 0.62 (SD 0.021) of the rumen NAN pool size for AN and SNAN treatments. The average AN concentration in the rumen was 5.5 mg/100 mL (SD 1.42). Passage rate of rumen fluid fraction averaged 0.163/h (SD 0.0150).

Ammonia N Metabolism

The model fitted well to the observed pattern of [15]NEP data. The residuals were randomly distributed, with the exception of greater random variation for the later time points in rumen [15]NEP of AN (Figure 3). The rumen [15]NEP of AN decreased rapidly, and the pool size at 2 h after dosing was about 0.01 of the initial dose. The [15]NEP pattern of bacterial N followed an initial ascending phase, reaching a peak between 1.5 and 3 h, and then declined steadily until the end of the sampling period. Initial uptake of AN was rapid, as approximately 0.16 of the initial dose appeared immediately in the bacterial N pool.

Metabolism of Soluble N

The fit of the 3-compartment model describing ruminal SNAN metabolism was good, with some deviations between observed and predicted [15]NEP of AN and SNAN at later sampling points when the pool sizes were small (Figure 4). Bacterial [15]NEP increased rapidly after the SNAN dose, reaching the maximum from

1.6 to 3.1 h, and 0.81 of the maximum observed at the first sampling point at 0.1 h. It is unlikely that such rapid enrichment was due to microbial protein synthesis. A more likely explanation for the rapid microbial enrichment is adsorption of labeled soluble protein to bacterial cells or protein adsorption in combination with uptake of free AA and peptides to intracellular pools. Therefore, an initial value for bacterial [15]NEP was estimated as an adjustable parameter of the model. The first-order disappearance rate from the SNAN pool was 0.52/h (SD 0.071). To facilitate comparisons between AN and SNAN treatments, the observations were scaled according to differences in ^{15}N dose (Figure 5). Ammonia [15]NEP was much greater for AN than for SNAN during the first 4 h. Interestingly, the residuals (observed − predicted) were rather similar among samples taken 10 h after dosing. Bacterial [15]NEP values were greater for SNAN treatment than for AN treatment during the first 4 h after dosing, but thereafter the values remained similar for both treatments.

Compartmental Models

All 6 parameter estimates for the AN treatment were different from 0 ($P < 0.01$), and FSD was below 0.5 (Table 4). Ammonia N disappeared rapidly by absorption and passage (ka_AN) or by bacterial uptake (ks_BN). Because of the instant uptake of AN by bacteria,
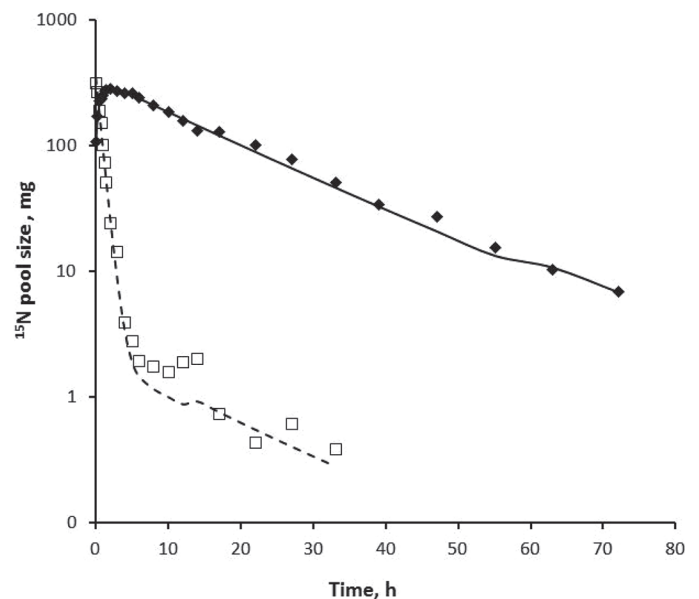
Figure 3. Predicted and observed pools sizes of ammonia ^{15}N and bacterial ^{15}N in the rumen in mg (average values from 4 animals), for ammonia N treatment. For ammonia ^{15}N, the predicted pool size is the dashed line, and the observed values are denoted by □; for bacterial ^{15}N, the predicted pool size is the solid line, and the observed values are denoted by ◆.
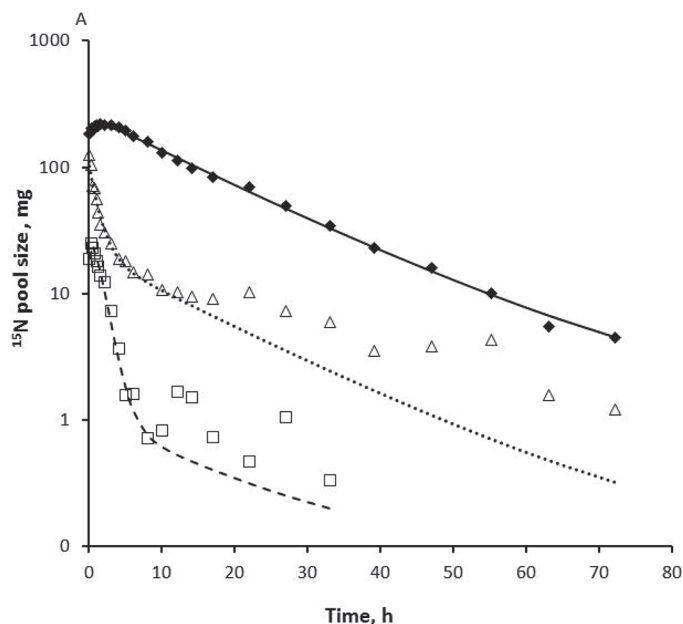
Figure 4. Predicted and observed pools sizes of ammonia ^{15}N, bacterial ^{15}N, and soluble non-ammonia ^{15}N in the rumen in mg (average values from 4 animals), for soluble N (SNAN) treatment. For ammonia ^{15}N, the predicted pool size is the dashed line, and the observed values are denoted by □; for bacterial ^{15}N, the predicted pool size is the solid line, and the observed values are denoted by ◆; for soluble non-ammonia ^{15}N, the predicted pool size is the dotted line, and the observed values are denoted by Δ.

initial bacterial N pool was an adjustable parameter. The instant bacterial uptake of AN (AN_BN) represented proportionally 0.16 of the AN dose (Table 4).

Table 4. Estimated parameter values for the ammonia N model

Parameter	Estimate[1]	FSD[2]	SD[3]
ka_AN,[4] per h	0.469	0.248	0.208
ks_BN,[5] per h	0.889	0.081	0.233
F_i,[6] mg/h	0.0410	0.348	0.082
kr_BN,[7] per h	0.00670	0.099	0.004
kp_BN,[8] per h	0.0612	0.036	0.012
AN_BN,[9] g/kg	156	—	60.6

[1]Estimates are means of 4 observations derived from fitting the model to data using WinSAAM software (version 3.0.7; http://www.winsaam.org/).

[2]FSD = fractional SD. All parameter estimates were significantly different from 0 ($P < 0.01$).

[3]SD between animals.

[4]ka_AN = rate of ammonia N disappearance from the reticulo-rumen (absorption + passage).

[5]ks_BN = rate of ammonia N synthesis to bacterial N.

[6]F_i = external input flux of ammonia N and recycled urea N.

[7]kr_BN = rate of bacterial N recycling through ammonia N.

[8]kp_BN = passage rate of bacterial N from the reticulo-rumen to the omasal canal.

[9]AN_BN = proportion of ammonia N dose instantly taken up by bacteria.

Parameter estimates describing ruminal metabolism of SNAN are presented in Table 5. The FSD estimates for adjustable variables were small, except for F_i (0.45), which describes external ^{15}N output from the diet and recycled urea. The instant uptake of SNAN to bacterial N (SNAN_BN) represented proportionally 0.56 of the SNAN dose; that is, 9.2 g of SNAN was rapidly taken up by the bacteria. After the rapid initial uptake, the rate of direct uptake of SNAN (0.273; ks_SNAN) was lower than that of SNAN degradation to AN (0.460; kd_SNAN). On average, proportionally 0.078 (BN_SNAN; FSD 0.067) of bacterial N was released to the SNAN pool during the preparation of bacterial sample. Passage rates of bacterial N were similar between AN and SNAN treatments (0.061 vs. 0.065/h, respectively). The rate of bacterial N recycling was less than 0.01/h for both AN and SNAN treatments.

Nitrogen Fluxes

The total entry rate of ruminal AN, estimated according to Nolan and Leng (1974), which represents the daily amount of N passing through the AN pool, was 320 (SD = 32.0) g/d for AN treatment, 0.58 (SD
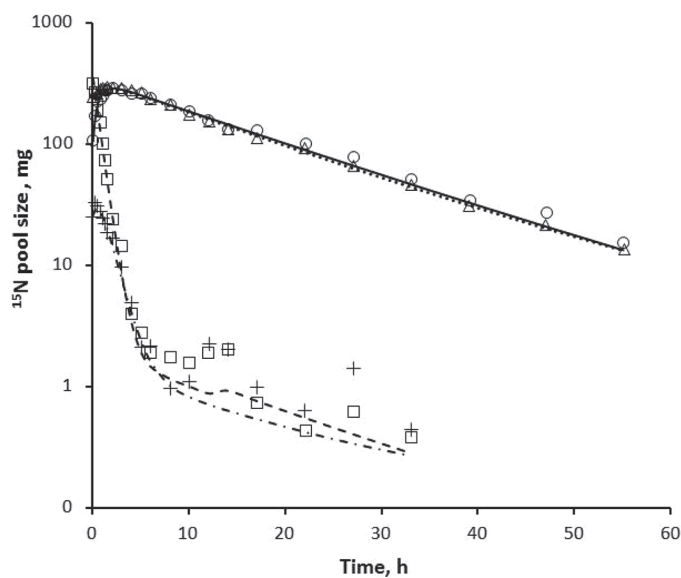
Figure 5. Comparison of predicted and observed pools sizes of ammonia ^{15}N and bacterial ^{15}N in the rumen in mg (average values from 4 animals), for ammonia N and soluble N (SNAN) treatments. To facilitate comparison between treatments, the data for SNAN treatment were scaled up to compensate for lower ^{15}N dose. For ammonia ^{15}N treatment, the predicted ammonia N pool size is the dashed line, and the observed values are denoted by □; the predicted pool size for bacterial ^{15}N is the solid line, and the observed values are denoted by ○. For SNAN treatment, the predicted pool size of ammonia ^{15}N is the dashed and dotted line, and the observed values are denoted by +; the predicted pool size for bacterial N is the dotted line, and the observed values are denoted by Δ.

Table 5. Estimated parameter values for the rapeseed meal soluble NAN model

Parameter	Estimate[1]	FSD[2]	SD[3]
ka_AN,[4] per h	0.469	—	0.208
ks_BN,[5] per h	0.889	—	0.233
ks_SNAN,[6] per h	0.273	0.257	0.131
kd_SNAN,[7] per h	0.460	0.112	0.206
F_i,[8] mg/h	0.110	0.449	0.070
SNAN_BN,[9] g/kg	0.561	—	0.084
kr_BN,[10] per h	0.00479	0.070	0.0003
kp_liquid,[11] per h	0.163	—	0.015
kp_BN,[12] per h	0.0646	0.020	0.017
BN_SNAN[13]	0.0782	0.067	0.012

[1]Estimates are means of 4 observations derived from fitting the model to data using WinSAAM software (version 3.0.7; http://www.winsaam.org/).

[2]FSD = fractional SD.

[3]SD between animals.

[4]ka_AN = rate of ammonia N disappearance from the reticulo-rumen. A fixed value derived from the ammonia N model.

[5]ks_BN = rate of ammonia N synthesis to bacterial N. A fixed value derived from the ammonia N model.

[6]ks_SNAN = rate of direct uptake of soluble NAN by bacteria.

[7]kd_SNAN = rate of degradation of soluble NAN to ammonia N.

[8]F_i = external input flux of ammonia N and recycled urea N.

[9]SNAN_BN = proportion of soluble NAN pool instantly taken up by bacteria.

[10]kr_BN = rate of bacterial N recycling through ammonia N.

[11]kp_liquid = rate of liquid matter outflow from the reticulo-rumen to the omasal canal estimated using LiCoEDTA.

[12]kp_BN = passage rate of bacterial N from the reticulo-rumen to the omasal canal.

[13]BN_SNAN = proportion of soluble NAN released from bacterial N.

= 0.072) of the daily N intake. Irreversible loss, which represents the fraction of the total flux leaving and not re-entering the rumen, was 237 (SD = 10.8) g/d. Recycling, calculated as a difference between the total flux and irreversible loss, was 0.26 (SD = 0.046) of the flux.

The net losses of AN from the rumen via absorption and outflow were smaller ($P < 0.01$) for SNAN treatment compared with AN treatment (Table 6). Direct uptake of SNAN by microbes was considerably greater than that of AN (561 vs. 156 mg/g, respectively). Intraruminal recycling of bacterial N represented a rather small proportion of total N flux (63 to 74 mg/g). Outflow of NAN from the rumen was greater ($P < 0.01$) for SNAN than for AN treatment, reflecting numerically greater bacterial N flow and escape of SNAN.

DISCUSSION

Model Development

Because of the rapid fractional disappearance of labeled AN after dosing, modeling intraruminal recycling of N is challenging, due to the low APE of AN 3 to 4 h after dosing. As discussed in our previous paper (Ahvenjärvi et al., 2018), diets based on grass silage and grain typically have positive $\delta^{15}N$ (e.g., Cheng et al., 2011). Delta ^{15}N represents the relative difference in ^{15}N abundance between biological material and atmospheric N [$\delta^{15}N$, ‰ = 1,000 × (s − a)/a, where s is ^{15}N abundance in a biological sample and a is ^{15}N abundance in atmospheric N]. Positive residuals were consistently observed for both AN and SNAN treatments in samples taken 2 to 3 h after the meals (e.g., 12, 14, and 27 h post-dose), probably arising from the contribution of positive dietary $\delta^{15}N$ to rumen APE of AN. Rumen microbes discriminate against ^{15}N (Wattiaux and Reed, 1995), which, together with positive $\delta^{15}N$ in the diet, can cause large relative deviations between observed and predicted ^{15}NEP of AN at late sampling times. Although urinary N is typically depleted in ^{15}N relative to the diet, $\delta^{15}N$ of urinary N was positive in cows fed grass silage–based diets (Cheng et al., 2011). To account for the contributions of small positive $\delta^{15}N$ from the diet, discrimination of microbes against ^{15}N

Table 6. Estimated transfers of 1,000 mg of ammonia N (AN) and soluble non-ammonia N (SNAN) from the reticulo-rumen based on model simulations

Item	AN	SNAN	SED[1]	P-value
Disappearance[2] of AN, mg	308	110	32.6	<0.01
Direct AN uptake to BN,[3] mg	156	—	60.6[4]	—
AN synthesized to BN, mg	611	200	86.1	0.02
Direct SNAN uptake to BN, mg	—	561	84.4[4]	—
SNAN synthesized to BN, mg	—	116	43.7[4]	—
Recycling of BN, mg	74	63	20.0	0.60
Outflow of BN, mg	692	814	41.6	0.61
Outflow of SNAN, mg	—	76	34.9[4]	—
Outflow of NAN, mg	692	890	32.6	<0.01

[1]SE of difference, unless otherwise stated.

[2]Includes ammonia N absorption and outflow from the rumen.

[3]BN = bacterial N.

[4]SD between animals.

and urea N recycling, a constant influx parameter was included in the model (F_i, Figure 1).

In the SNAN model, the parameter estimates derived from the AN model for the rate of AN disappearance and uptake by microbes were used as fixed parameters. It can be postulated that the kinetics of extracellular AN are not influenced by the origin of the molecules. The initial model that assumed recycling of bacterial N to the SNAN pool underpredicted [15]NEP of the SNAN pool between 8 and 10 h post-dose. This observation suggests that predicted recycling was unrealistically high (1.4 times higher than the predicted bacterial N flow). In the final model, an assumption was made that a fraction of the SNAN pool was bacterial N—that is, the SNAN pool comprised extracellular AA, peptides, and protein that were partly of bacterial origin. This assumption resulted in much better agreement between the predicted and observed patterns of SNAN pool size. On average, proportionally 0.078 of [15]NEP of bacterial N was found in the SNAN pool. This parameter estimate was associated with small FSD (0.067), and between-animal standard deviation for the estimate was also small (0.012). Such a consistency among these estimates tends to indicate that the proportion of bacterial N associated with the SNAN pool is a well-distinguished and biologically relevant observation. It can be speculated that ^{15}N from labeled SNAN adsorbed to cell surfaces or taken up to intracellular pools was released during processing of the samples. Bacterial cell surfaces can be damaged due to centrifugal compaction (Peterson et al., 2012). It is also possible that centrifugation force was not sufficient for complete recovery of bacterial N in the pellet. In the study of Hsu and Fahey (1990), increasing centrifugation force increased bacterial DM in rumen fluid, although not significantly.

Ammonia N Metabolism

A considerable proportion (0.16) of the AN dose was instantly absorbed into bacterial N, as indicated by the initial condition (at 0 h) estimated for bacterial N pool size (Table 4). The initial condition represents instant uptake of extracellular AN to the microbial N pool that could not be predicted with the current model. The process of instant uptake occurred at such a high rate that it was complete by 0.1 h. In a previous study that involved a similar dose of labeled AN into the rumen of lactating dairy cows (Ahvenjärvi et al., 2018), no initial condition was required to represent instant microbial uptake of AN. An obvious explanation for this difference is related to lower rumen AN concentration in the current study (5.5 mg/100 mL) compared with the previous one (7.4 mg/100 mL; Ahvenjärvi et al., 2018). When rumen AN concentrations are low, rumen

bacteria absorb extracellular AN efficiently to maintain sufficient intracellular AN concentrations (Russell and Strobel, 1987). Efficient capture of AN from extracellular space has been reported in previous studies that supplemented increasing levels of urea N into the rumen (Marini and Van Amburgh, 2003; Ahvenjärvi and Huhtanen, 2018). In the study of Ahvenjärvi and Huhtanen (2018) rumen microbes absorbed AN from extracellular space so efficiently that rumen AN concentrations did not respond to increasing levels of urea N administration up to 49 g/d (CP concentration of 140 g/kg of DM). The highest level of urea N administered into the rumen, 66 g/d (CP concentration of 145 g/kg of DM), sharply increased AN concentrations in the rumen. Similarly, in studies by Slyter et al. (1979) and Marini and Van Amburgh (2003), increasing the CP content of the diet was found to increase rumen AN concentrations in a curvilinear manner, with a smaller slope at lower diet CP concentrations. Higher levels of urea N administration into the diet sharply increased rumen AN concentrations in both studies. These observations further support the theory that rumen microorganisms very efficiently capture AN from the extracellular space if rumen AN concentrations are low.

Metabolism of Soluble N

Disappearance of soluble protein N in the rumen was characterized by 2 discernible steps: (1) an instant adsorption onto bacterial cells that was too fast to be measured with the current methodology, and (2) slower first-order rates attributable to SNAN degradation to AN (kd_SNAN), incorporation into microbial N (ks_SNAN), and outflow from the rumen (kp_liquid). In the current study, instant adsorption of SNAN onto bacterial cells was proportionally 0.56 of SNAN dose at 0.08 h. Some evidence suggests that the capacity of rumen microbial uptake and degradation of soluble protein is limited. Using ^{14}C-labeled rubisco protein, Nugent and Mangan (1981) observed rapid adsorption of soluble protein to bacterial cells, whereas protozoa did not appear to play an active role in initial protein uptake. The rate of ruminal proteolysis studied in vitro exhibited typical Michaelis–Menten kinetics. Decreasing initial velocities with increasing concentrations of Rubisco protein indicated that proteolytic activity could be limited and could be saturated with sufficiently large doses of protein. In addition, decreasing proportions of protein adsorbed onto particulate matter with increasing amounts of protein indicated that the sites of rapid adsorption of soluble protein could be saturated (Nugent and Mangan, 1981).

Volden et al. (2002) administered increasing levels of soluble extracts of silage (21, 27, and 32 g of NAN)

as a pulse dose into the rumen of lactating dairy cows and observed a linear decrease in estimates of ruminal degradation rate (2.52, 2.06, and 1.71/h, respectively). The current estimates of 9.2 g of SNAN for the instant uptake represented, proportionally, 0.039 of the rumen bacterial N pool. This estimate is considerably higher than that reported by Wallace (1985) based on an in vitro study. The maximum casein adsorption capacity of rumen bacteria was 10 mg of casein per g of bacterial protein (Wallace, 1985), which is proportionally 0.01 of the bacterial N pool size.

Assuming that rumen bacteria have limited proteolytic activity and capacity for immediate adsorption of soluble protein, it can be speculated that ruminal protein metabolism is dose dependent if supplied as sufficiently large meals. When fed as TMR, protein intake occurs as multiple meals throughout the day (9.5 meals/d; Hart et al., 2014). Supplementation of protein-enriched concentrates as separate infrequent meals, as by using automatic out-of-parlor systems, may deliver protein as larger doses into the rumen. If a cow consumes 500 g/d of protein N as either 10 or 2 meals per day, each meal supplies either 50 or 250 g of protein N, respectively. Bacterial capacity to immediately adsorb 10 g of protein N to the bacterial N pool represents 0.2 and 0.04, respectively, of protein N entering the rumen when fed as either 10 or 2 meals a day. If limited proteolytic activity decreases the rate of protein degradation when fed as 2 large meals daily, these mechanisms combined might enhance ruminal escape of RUP.

Compartmental Models

In the present study, the recovery of SNAN as NAN flow entering the omasal canal was greater than that of AN (890 vs. 692 g/kg). In a previous study (Ahvenjärvi et al., 2018), the recovery of SNAN extracted from grass silage was higher than that of AN (503 vs. 387 g/kg). These differences were partly due to the greater capture of N by rumen microbes and partly due to the outflow of SNAN from the rumen. This suggests that the effect of N solubility on MP concentration is less than estimated by most feed protein evaluation systems. For example, the NRC (2001) protein evaluation system is based on ruminal protein degradation estimated using in situ methodology. In situ determinations of protein degradation are flawed because of several inherent problems, including (1) the assumption that proteins, peptides, and AA in the soluble fraction are completely degraded in the rumen; (2) the physical restriction of feeds within the bag from microbial interaction and digestion; and (3) the imprecise quantitation of microbial contamination of the undigested residues (Broderick

and Cochran, 2000). Despite suggestions to standardize the in situ method, time-series analysis of the data collected suggests that standardization does not appear to be occurring in practice (Liebe et al., 2018).

The assumption that the SNAN fraction is completely degraded is probably the most serious limitation of the in situ method, as the results of the present study and many previous studies have demonstrated (for discussion see Ahvenjärvi et al., 2018). Some recently introduced protein evaluation systems attempt to overcome this shortcoming by assuming a constant rate of degradation for the SNAN fraction. Van Duinkerken et al. (2011) assumed that the SNAN fraction is degraded at a constant rate of 2.0/h, whereas the NorFor system (Volden, 2011) assumes a corresponding value of 1.5/h. The updated Cornell Net Carbohydrate and Protein System uses values of 2.0/h for PA_1 (soluble NPN) and 0.10 to 0.40 for PA_2 (soluble true protein N), as described by Van Amburgh et al. (2015). The first-order disappearance rate of labeled RSM SNAN ($PA_1 + PA_2$ in the CNCPS system) during the first 1.5 h after dosing was on average 0.52/h (SD = 0.070). This rate aggregates uptake to the microbial N pool, degradation to AN, and passage from the rumen, suggesting that degradation rate of SNAN of RSM is rather slow. In vitro degradation rate of soluble protein from rapeseed meal and cake was slower than that of other protein feeds or forages (Hedqvist and Udén, 2006). This may also explain the lack of responses in duodenal NAN flow to reduced ruminal degradability of RSM obtained by a treatment with acetic acid (Robinson et al., 1994) or by heat treatment (Ahvenjärvi et al., 1999); that is, true differences in ruminal protein degradability are smaller than those determined via in situ incubations.

Soluble NAN from rapeseed meal (the current study) and from formic acid–treated grass silage (Ahvenjärvi et al., 2018) were used more efficiently for microbial protein synthesis than AN, indicating stimulation of microbial N synthesis when preformed AA were supplied to microbes. This is consistent with several previous studies (Rooke and Armstrong, 1989; Chikunya et al., 1996; Walker et al., 2005; Brito et al., 2007). On the other hand, strong evidence from the meta-analysis of Santos et al. (1998) and Ipharraguerre and Clark (2005) indicates that the efficiency of microbial protein synthesis decreases when highly degradable protein sources such as solvent-extracted soybean meal (**SBM**) were replaced with RUP sources. Broderick and Reynal (2009) gradually replaced solvent-extracted SBM with lignosulphonate-treated SBM and urea so that the estimated (NRC, 2001) dietary RDP and RUP concentrations were equal for all treatments. Microbial N and total NAN flow decreased without any changes in RUP flow with increasing proportions of treated SBM. In

the study by Krizsan et al. (2017), gradual replacement of crimped barley with heat-treated RSM increased NAN flow to the omasal canal, but the increase was proportionally only 0.60 of feed N flow due to reduced microbial protein synthesis.

Ipharraguerre and Clark (2005) suggested that shortage of AA, peptides, or AN can depress microbial N synthesis with high-RUP diets, but in the studies of Broderick and Reynal (2009) and Krizsan et al. (2017), the diets containing treated protein supplements increased ruminal AN concentrations. Stimulation of microbial protein synthesis and increased escape of SNAN can result in underestimation of MP in untreated RSM compared with heat-treated RSM in the current feed protein evaluation systems. Similar milk and milk protein yield responses to untreated and heat-treated RSM in the meta-analysis by Huhtanen et al. (2011) support this hypothesis.

CONCLUSIONS

Utilization of SNAN from rapeseed meal in the rumen was higher than that of AN, as indicated by greater estimated NAN flow from the rumen and smaller absorption as AN. Adsorption to microbial cells and uptake to intracellular pools rather than degradation to AN explained rapid disappearance of labeled SNAN from rumen fluid. More studies with incremental levels of soluble protein are required to quantify the effects of SNAN adsorption onto and uptake into bacterial cells on post-ruminal RUP supply.

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