Effect of method of conservation of timothy on endogenous nitrogen flows in lactating dairy cows

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ABSTRACT

The effect of the method of conservation of forage on endogenous N (EN) secretion was studied using a ¹⁵N isotope dilution technique in 4 lactating Holstein cows selected from a replicated 3 × 3 Latin square. Cows were equipped with ruminal, duodenal (n = 4), and ileal (n = 2) cannulas. Diets comprised 44% concentrate plus first-cut timothy conserved either as hay or as restrictively (formic) or extensively (inoc) fermented silage. Crude protein contents of hay, formic, and inoc averaged 10.4, 13.6, and 14.8%, respectively. Total EN flow and free EN at the duodenum were increased with hay compared with silages but were similar when expressed as proportion of duodenal N flow, with total EN flow averaging 25.8, 23.9, and 23.9% for hay, formic, and inoc, respectively, and free EN at the duodenum averaging 11.5, 9.8, and 9.7% for hay, formic, and inoc, respectively. Flow of bacterial N at the duodenum originating from an endogenous source tended to be higher with inoc compared with formic. Overall, the proportion of bacterial N derived from endogenous sources and urea was similar between treatments, averaging 23 and 15%, respectively. In the feces, flow of EN was similar across treatments and averaged 31% of total fecal N. More than 70% of fecal EN originated from undigested secretions into the forestomach. Absorption of N from the forestomach tended to increase for silages compared with hay. In conclusion, EN represented an important fraction of N flowing at the duodenum and in the feces. The free EN and the total EN at the duodenum were altered by the different methods of forage conservation studied. Estimation of true dietary N supply and requirements of the dairy cow should allow for endogenous N flows and losses.

Key words: endogenous nitrogen, dairy cow, forage conservation, stable isotope

INTRODUCTION

Three major sources contribute to the N flow at the duodenum: microbial CP (MCP), RUP, and endogenous N (EN) secretions (NRC, 2001). Endogenous N flow has important implications for N transactions in animals because this involves recycling of anabolic N, such as AA N, between the body tissues and the gut lumen and does not make a net addition to N supply to the animal (Larsen et al., 2000; Sandek et al., 2001). In contrast, other sources of N normally considered as end products of host metabolism, such as urea, if reutilized within the gut lumen, would be considered a potential gain (Lapierre and Lobley, 2001). Often, these different sources and fates of N are not clearly resolved by the techniques usually employed to quantify the so-called EN secretions. Although it is recognized that EN at the duodenum needs to be quantified (NRC, 2001; INRA, 2004), most of the published data partition duodenal protein flow only between MCP and RUP, the latter obtained by difference between duodenal protein flow and MCP. Inevitably this will overestimate the true protein supply from undegraded feed protein. Also, the scarcity of data on EN in ruminants has constrained models to use fixed factors (e.g., based on DMI: NRC, 2001) to estimate for EN flows, failing to account for differences that may occur because of the nature of the ration, as acknowledged in pig nutrition (Jansman et al., 2002). Currently, stable isotope dilution techniques are the best approaches; with these, it was estimated that in dairy cows the EN contribution, excluding urea, could represent as much as 15% of the duodenal flow, with this partitioned between free EN and EN incorporated into MCP (Ouellet et al., 2002).

The form in which the forage is stored and fed directly affects the flow of N at the duodenum (McDonald et al., 1991). For example, conservation either as hay or as silages treated with formic acid or inoculated with lactic acid bacteria affects the distribution of N within the forage (NRC, 2001) and leads to altered total N flow at the duodenum (Martineau et al., 2007). In hay,

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NPN represents approximately 40% of total N and remains mostly unchanged during storage. Addition of inoculants of homofermentative lactic acid bacteria to silage results in greater fermentation and proteolysis with more ammonia N release than when fermentation is restricted with formic acid treatment, which reduces the proportion of NPN, ammonia, and free AA released in silage and thereby limits N degradation in the rumen (McDonald et al., 1991; Nagel and Broderick, 1992; Martineau et al., 2006). In addition, the preservation of water-soluble carbohydrates is an important feature of both hay and restrictively fermented silages as opposed to extensively fermented silages, where they are fermented to lactic acid and VFA (McDonald et al., 1991). All of these characteristics related to forage conservation have the potential to affect rumen environment and, consequently, urea recycling, bacteria synthesis, and EN movement. In addition, the particles length of the forage can also increase cell sloughing (Greenwood et al., 1997; Sandek et al., 2001) and therefore increase the contribution of cells and enzyme secretions to duodenal flow (Ørskov et al., 1986; Swanson et al., 2004).

Because sources of N and energy in the rumen directly affect duodenal N flow and because the abrasive action of the digesta can affect cell desquamation (Kennedy and Milligan, 1980; Sandek et al., 2001), we hypothesized that the mode of conservation of the forage will alter EN flows at the duodenum and ileum and in the feces. Therefore, the current study examined how EN varied when timothy, contributing more than 50% of the ration, was harvested as hay, restrictively fermented silage, or extensively fermented silage. This is part of a larger study designed to understand the complex interplays between N sources (including diet, recycled urea, and EN) within the digestive tract and their effect on true availability of N to the cow (Martineau et al., 2006, 2007).

MATERIALS AND METHODS

Forage Production, Cows, and Diets

The experimental conditions met the guidelines of the Canadian Council on Animal Care (1993) and were approved by the Animal Care Committee of the Dairy and Swine Research and Development Centre (Sherbrooke, Quebec, Canada).

A more exhaustive description of the procedures used in the present experiment was published previously (Martineau et al., 2007). Briefly, 3 forages were prepared from the same timothy (*Phleum pratense* L.) sward. The first forage was conserved as small rectangular bales of hay, the second was conserved as restrictively fermented silage (referred to as formic; 85% formic acid

at a rate of 6 L/t of fresh forage), and the third forage was an extensively fermented silage (referred to as **inoc**; *Lactobacillus plantarum* LPH-1 and *Pediococcus cerevisiae* PCH-3 at final application rate of 1.25×10^5 cfu/g of fresh forage).

Six Holstein primiparous lactating cows had been surgically fitted with both ruminal and duodenal cannulas (closed T-shaped, 10 to 15 cm distal to the pylorus) and used in a replicated 3×3 Latin square with 35-d periods (Martineau et al., 2007). Two cows were also fitted with open-T ileal cannulas. Each of the 3 forage forms provided 56% of DM and was supplemented with a concentrate containing, on a DM basis, 76% ground shelled corn, 13% soybean meal, 5% calcium salt of fatty acids (Megalac, Church and Dwight Co. Inc., Princeton, NJ), and 6% mineral and vitamin premix (Martineau et al., 2007). Chromium sesquioxide (Cr_2O_3), used as indigestible marker, was mixed with the concentrate before pelleting to provide a daily intake of approximately 15 g of Cr_2O_3 /cow. Rations containing 12.5, 14.6, and 15.2% CP for hay, formic, and inoc, respectively, were offered at 95% of ad libitum DMI determined during the first week of period 1. All diets were fed as TMR and distributed every other hour in 12 equal meals daily (Ankom Technology, Fairport, NY). The hay was coarsely chopped to facilitate delivery through the automated feeders. To prevent ruminal disturbance, in both the formic and inoc diets 1 kg (as-fed basis) of unchopped hay was offered daily. Dry matter content of silages was estimated weekly to deliver constant DM throughout the experiment (DMI = 17.5 kg/d; SD 2.7). Amount of feed offered and orts, when present, were recorded daily. Samples of TMR, feed ingredients, and orts were sampled daily and kept frozen at -20°C before being combined by period for further analyses. Animals were housed in individual tie stalls equipped with rubber mats and had free access to water. Cows were milked twice daily, at 0700 and 1900 h, and the production was recorded. Live weight was recorded at the beginning and end of each period.

Data and Sample Collection

The first 16 d of each period were allowed for adaptation to the diet. Total collection of feces and urine was performed from d 16 to 22 using the methodology previously reported (Martineau et al., 2007). Representative daily samples were pooled across days by animal and period before analysis for DM, N, and urinary purine derivatives. Urea kinetics were determined between d 18 and 22 on the 6 cows (Martineau et al., 2007). Polyvinyl catheters were inserted into 1 jugular vein on d 18. On d 19, cows were infused (0.5 mmol/h) in the jugular vein with $[^{15}\text{N}_2]\text{urea}$ (98% purity, Cambridge Isotope

Laboratories Inc., Andover, MA) for 72 h. Duodenal digesta, fecal grab samples, and spot samples of urine were collected at 0900 and 1000 h on d 18 (natural abundance) and on d 22. Because of the prohibitive cost of stable isotopes, only 4 cows (mean $\pm$ SD; 623 $\pm$ 63 kg of BW; 104 $\pm$ 28 DIM) were selected to evaluate EN flows. From d 27 to 35, L-[^{15}N]leucine (99 atom %; ACP, Montreal, Canada) was infused continuously (0.45 mmol/h) in these 4 cows with peristaltic pumps (Masterflex pump, model 7523-30, Cole Parmer Instrument Co., Niles, IL) into one of the jugular veins to label and estimate EN secretions. Samples of ruminal and intestinal mucosa, duodenal and ileal digesta, feces, and urine were taken at 0900, 1100, 1300, and 1500 h on d 34 and at 0800, 1000, 1200, and 1400 h on d 35. Endometrial forceps were used for the ruminal mucosa biopsy (approximately 10 mg) and an optical fiber endoscope (Olympus CF type P10L, Olympus Co. Ltd., Tokyo, Japan) was introduced approximately 20 to 30 cm distally from the opening of the duodenal cannula for the intestinal mucosa biopsy (3 to 5 subsamples, each of approximately 0.5 mg on wet weight basis). The samples collected were immediately rinsed with saline (0.9% NaCl) and frozen at -20°C until further analysis. To preserve bacterial integrity during storage, duodenal and ileal subsamples were formolized (300 mL plus 75 mL of formol physiological saline solution) whereas fecal subsamples were diluted (25 g plus 140 mL of distilled water) before 40 mL of formol physiological saline solution was added. Urine (100 mL) was preserved with 2 mL of sulfuric acid (50% vol/vol) and frozen at -20°C until chemical determination. Duodenal and ileal bacterial pellets were prepared from approximately 100 g of thawed formolized digesta for each hourly sample by adapting the method described by Legay-Carmier and Bauchart (1989). Briefly, each sample was homogenized in 300 mL of NaCl 0.9% for 2 min, then the homogenate was allowed to soak for 3 h at 4°C before being stomached for 5 min and subsequently filtrated on a 0.25-mm sieve. The filtrate was centrifuged at $800 \times g$ for 10 min at 4°C . The supernatant was centrifuged twice at $800 \times g$ for 10 min and then the resulting supernatant was centrifuged at $9,900 \times g$ for 20 min at 4°C . The pellet was washed with 300 mL of sterile saline followed by centrifugation ($9,900 \times g$ for 20 min at 4°C). This washing was repeated once. The bacteria pellet was resuspended in 50 mL of sterile water, frozen at -20°C , and freeze-dried.

Chemical Analyses

Urea Kinetics. The ^{15}N -urea isotopomers from urine were prepared and analyzed as described by Saraseca et al. (1998). Urinary urea N excretion was esti-

mated from urinary volume collected on d 22 and urea concentration from a pooled sample from d 19 to 22. For bacteria isolation, approximately 100 g of thawed formolized duodenal digesta from each sample was prepared as described previously. The ^{15}N enrichment of bacteria was determined as described below. The rumen fluid was strained through 4 layers of cheesecloth and centrifuged at $20,000 \times g$ for 15 min. Ten milliliters of the supernatant mixed with 10 mL of saturated sodium tetraborate as the alkaline reagent were steam distilled using a Kjeltex apparatus (Tecator 1030) modified to allow collection of the distillate. Distillates were trapped using 15 mL of 0.05 N H_2SO_4 and dried. The dried ammonium salt and bacteria were analyzed for ^{15}N isotopic enrichment (**IE**) using a Europa 20:20 continuous-flow isotope ratio mass spectrometer (Europa Scientific, Crewe, UK) interfaced to a Roboprep CN elemental analyzer (Europa Scientific).

Endogenous N Secretions. The total N content of feed and orts samples was determined by thermal conductivity (Leco model FP-428 Nitrogen Determinator, Leco, St. Joseph, MI). The concentration of N in acidified urine samples was determined by micro-Kjeldahl analysis (method 955.04; AOAC, 1990). Fecal and digesta samples collected on d 34 and 35 were freeze dried, ground through a 1-mm screen Wiley mill (Arthur H. Thomas, Philadelphia, PA), and analyzed for analytical DM (135°C forced-air oven for 2 h for digesta samples or 70°C vacuum oven for 12 h for fecal samples). Ruminal and duodenal biopsies were freeze dried and homogenized with a mortar and pestle. For each animal, processed samples from the 2 morning collections were pooled, as were the samples from the 2 afternoon collections, to produce morning and afternoon composite samples ($n = 4$) to determine IE using an elemental analyzer (Carlo Erba Strumentazione NA 1500, Milan, Italy) coupled to an isotope ratio mass spectrometer (SIRA 12, VG Isogas, Middlewich, UK). Chromium in the pooled fecal samples collected during the N balance and in daily pools of feces and digesta collected on d 34 and 35 was measured by atomic absorption with an air-acetylene flame (Siddons et al., 1985).

Calculations

Urea kinetics were estimated using the model developed by Lobley et al. (2000). The EN secretions were calculated as presented in Ouellet et al. (2002), except that the proportion of duodenal bacteria originating from urea recycled to the rumen was directly estimated by the ratio of the IE (atom % excess) of bacterial N to urinary urea N from samples collected on d 22 at the end of the labeled urea infusion. Similarly, the

proportion of bacteria N originating from ruminal ammonia was determined using ^{15}N IE (atom % excess) of the ammonia in the rumen fluid over that of total bacteria N, also on samples collected on d 22 of each of the 3 periods. For the EN secretion flow at the ileum, the calculations were identical to calculations in the feces, except that the ending point was the ileal digesta rather than the feces. Based on the biopsies taken during d 34 and 35 of each period, the calculated EN is presented using 2 possible precursor pools: mucosa and combined. For mucosa, the IE of the rumen biopsy for the forestomach compartment and the IE of the intestinal mucosa for the intestine compartment were selected as representative of the EN secretions IE preduodenal and in the intestine, respectively. For the combined estimation, EN flows were based using the average IE of the corresponding gut mucosa and milk casein, the latter taken as representative of proteins exported from their tissue site of synthesis (e.g., digestive enzymes and mucin secretions within the gut; Lapierre et al., 2008).

Statistical Analyses

For each of the 2 methods (mucosa, combined), results were analyzed using the MIXED procedure of SAS (SAS, 2000). The statistical model included fixed effects of treatment and period and random effect of cow, with the exception of ileocecal data where period effect could not be included in the model because of the limited number of observations (2 cows). The effects of treatments were evaluated using preplanned orthogonal contrasts: hay vs. silages (formic and inoc) and formic vs. inoc. Significance was declared at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$. The difference between IE (average of d 34 and 35) of rumen biopsy, intestinal biopsy, and milk casein was evaluated using Bonferroni's multiple comparison tests.

RESULTS AND DISCUSSION

Urea Kinetics

Total urea production, measured in the 6 cows, increased ($P = 0.02$; Table 1) in cows fed inoc compared with those fed formic. The ruminal ammonia contributed predominantly to bacteria N (0.81, 0.81, and 0.87 for hay, formic, and inoc, respectively; SEM = 0.07). These proportions were unaffected by treatment but they are at the upper limit of values previously reported (Oldham et al., 1980; Koenig et al., 2000; Hristov et al., 2004). The high values reported in the present study could be partly attributed to efficient capture of ammonia resulting from restricted feeding of a low N diet at

frequent intervals. The contribution of urea to bacteria was also similar among treatment and averaged 0.15, 0.16, and 0.15 (SEM = 0.01; Table 1) for hay, formic, and inoc, respectively. These values are similar to the value of 0.19 reported for heifers fed a high forage diet (Marini and Van Amburgh, 2003) but lower than values reported in cows (0.38; Al-Dehneh et al., 1997) or steers (0.30; Wickersham et al., 2009) fed high concentrate diets. The relatively low values reported in the present study can be related to low OM available in the rumen, which has been shown to reduce urea recycling and capture into the rumen (Rémond et al., 1996). In the first estimation of EN (Ouellet et al., 2002), a fixed contribution of urea to bacteria N of 0.12, estimated from literature, had been used. A sensitivity analysis had been conducted and indicated that the EN flux at the duodenum could present an error of 12% if the estimated value deviates from 50% of the proportion of 0.12 (Zuur et al., 2001). Therefore, a direct measurement of the urea contribution to bacteria, which could differ between treatments, could improve the estimation of EN flows at the duodenum. Urea entry to the gut tended to be greater ($P = 0.10$; Table 1) for the inoc treatment. These results are in agreement with the higher ammonia concentration in the rumen fluid reported in cows fed the inoc treatment (Martineau et al., 2007). Urinary urea excretion and the return to the ornithine cycle were larger ($P < 0.05$; Table 1) for cows fed inoc compared with those fed formic. These results, similar to those reported for cows supplemented with urea (Lapierre et al., 2004), are in agreement with the reduction in protein solubilization measured when restricting silage fermentation with formic was compared with stimulating fermentation with inoc (Martineau et al., 2006). The lower effective degradability of CP in hay compared with silages (Martineau et al., 2006) did not, however, alter urea kinetics. The utilization of urea for anabolism (UA) was systematically greater than the estimated bacterial flow derived from urea; this difference is even larger when comparing UA and the digested bacteria. This has been reported with bacteria flow estimated indirectly (Marini and Van Amburgh, 2003) or directly (Wickersham et al., 2009), and could be related to uncertainties involved in the estimation of UA obtained by difference. Part of the discrepancy between UA and bacteria derived from urea may relate to the fact that a proportion of the anabolism process can also occur through protozoa growth. Also, absorbed ammonia could be used in transamination reactions and contribute to UA. Although the amount of urea N losses to feces was not different between treatments, the fractional transfer of the gut entry rate to fecal loss was larger ($P < 0.05$; Table 1) when cows were fed hay compared with the silages, probably as a consequence

Table 1. Effect of forage conservation on the proportion of ruminal ammonia and recycled urea-N incorporated into the bacteria N and urea N metabolism¹

Parameter ²	Treatment ³			SEM	Contrast	
	Hay	Formic	Inoc		Hay vs. formic and inoc	Formic vs. inoc
¹⁵ N enrichment of ruminal ammonia, ape	0.0206	0.0260	0.0190	0.0024	NS ⁴	0.02
¹⁵ N enrichment of bacteria, ape	0.0190	0.0210	0.0170	0.0015	NS	0.09
¹⁵ N enrichment of urinary urea, ape	0.1256	0.1283	0.1110	0.076	NS	NS
Proportion of bacteria N derived from ammonia	0.81	0.81	0.87	0.07	NS	NS
Proportion of bacteria N derived from urea	0.15	0.16	0.16	0.01	NS	NS
Urea kinetic variables, g of N/d						
UER	173.2	170.6	200.0	13.7	NS	0.02
GER	125.6	121.6	140.4	11.0	NS	0.10
UUE	49.0	48.9	59.6	5.7	NS	0.05
ROC	54.6	53.2	62.7	4.9	NS	0.04
UFE	14.4	12.2	13.6	1.5	NS	NS
UA	57.0	56.2	64.1	6.3	NS	NS
Fractional transfer						
UER to urine	0.277	0.287	0.298	0.025	NS	NS
UER to gut (1 – urine)	0.723	0.713	0.702	0.025	NS	NS
GER to ROC	0.437	0.438	0.448	0.019	NS	NS
GER to feces	0.115	0.102	0.095	0.007	0.03	NS
GER to UA	0.449	0.460	0.458	0.022	NS	NS

¹Least squares means and the highest SEM based on n = 5, 6, and 6 for hay, formic, and inoc, respectively.

²ape = atom percent excess (%); UER = production; GER = entry to gut; UUE = urinary urea excretion; ROC = return to ornithine cycle; UFE = losses to feces; UA = reuse for anabolism.

³Formic = 85% formic acid at a rate of 6 L/t of fresh forage; inoc = *Lactobacillus plantarum* LPH-1 and *Pediococcus cerevisiae* PCH-3 at final application rate of 1.25×10^5 cfu/g of fresh forage.

⁴NS = $P > 0.10$.

of greater supply of fermentable energy in the form of fiber to the lower digestive tract (Lapierre and Lobley, 2001).

Effect of Forage Conservation Mode on Endogenous N Secretion

The effect of precursor pool selection will be discussed later. The following section refers to data calculated with the mucosa method when appropriate and based on 4 animals. The N intake and total N flow at the duodenum were greater for cows fed silages compared with those fed hay (Table 2). For cows fed hay, duodenal N flux exceeded N intake (Table 2), indicating an additional net inflow of N from urea or EN or both. In the present study, no differences in urea N recycled to either the total gut (Table 1) or the forestomach (Table 3) were observed between treatments. In contrast, flow of free EN at the duodenum was greater ($P = 0.02$) for cows fed hay. This may be attributed to a greater rate of cell sloughing caused by the longer particles present in hay (Greenwood et al., 1997; Sandek et al., 2001), or by an increase in the contribution of cells and enzyme secretions from the omasal and abomasal compartments (Ørskov et al., 1986; Swanson et al., 2004), or both. The proportion of free EN at the duodenum averaged 10% of total duodenal N, similar to

the 13% reported by Marini et al. (2008) based on a meta-analysis approach. Undigested N flow from feed sources did not differ ($P > 0.10$) between treatments and represented 28% of the N intake (Table 2). The amount of bacterial N derived from feed tended to be reduced ($P = 0.06$) for cows fed silages, especially with formic treatment. Bacterial N derived from endogenous and urea was similar between treatments, averaging 23 and 15%, respectively. Interestingly, the contribution of EN to bacterial N was greater than that from urea. This may affect ration formulation, especially for those schemes based on AA profiles. Overall, total EN flow at the duodenum (sum of free EN + EN incorporated into bacteria) tended ($P = 0.07$) to be higher in cows fed hay compared with those fed silages but was similar when expressed as proportion of duodenal N flow, averaging 25.8, 23.9, and 23.9% of duodenal N flows for hay, formic, and inoc, respectively. From a meta-analysis, Marini et al. (2008) estimated that 39% of total N available in the rumen was derived from EN sources, including recycled urea. This compares with an average of 42% across treatments obtained in the present study, based on the sum of EN contribution to the forestomach (145 g) and urea contribution (urea N recycled to the forestomach = 57 g) relative to the amount of degraded dietary N (279 g/d). A fraction of this overestimation may be attributed to the fact

Table 2. Flows of N across the digestive tract estimated using the gut mucosa (mucosa) or the average enrichment of the gut mucosa and milk casein (combined) as representative of the endogenous secretions in cows fed hay, formic acid-treated silage (formic), or inoculated silage (inoc)^{1,2}

Parameter, g of N/d	Mucosa			Combined			SEM	Contrast	
	Hay	Formic	Inoc	Hay	Formic	Inoc		Hay vs. formic and inoc	Formic vs. inoc
Intake	350	403	414	350	403	414	34	0.001	NS ³
Duodenal digesta	402	376	410	402	376	410	41	0.03	0.001
Undigested feed	100	113	118	118	128	135	17 (17)	NS (NS)	NS (NS)
Free endogenous N	46	35	40	28	21	22	2 (2)	0.02 (0.02)	NS (NS)
Bacterial N	257	228	253	257	228	253	26	0.001	0.001
From feed	163	138	156	185	160	181	18 (18)	0.06 (0.04)	0.06 (0.02)
From endogenous N	57	52	58	35	31	32	4 (3)	NS (NS)	0.07 (NS)
From urea N	38	37	40	38	37	40	7	NS	NS
Total endogenous N in duodenal digesta	104	87	97	64	51	55	4 (4)	0.07 (0.04)	NS (NS)
Feces	125	123	135	125	123	135	13	0.03	0.001
Undigested duodenal digesta ⁴	116	112	123	119	116	127	13 (13)	NS (NS)	NS (NS)
Endogenous N from undigested secretions into intestine	11	10	13	7	6	8	4 (3)	NS (NS)	NS (NS)
Endogenous N from undigested secretions into forestomach	31	26	29	19	16	17	2 (2)	NS (NS)	NS (NS)
Total endogenous N in feces	41	36	42	25	23	24	4 (3)	NS (NS)	NS (NS)

¹Least squares means and the highest SEM based on n = 3, 4, and 4 for hay, formic, and inoc, respectively.

²Contrast and SEM are presented as unique value for mucosa and combined methods when identical; if different, combined value is presented in parentheses.

³NS = $P > 0.10$.

⁴Undigested duodenal digesta = total fecal N – endogenous N from undigested secretions into intestine.

that EN estimates in the current model are from the duodenum, which include EN secretions from omasal and abomasal sources.

Total fecal N loss was greater for cows fed inoc compared with those fed formic ($P = 0.001$; Table 2). Fecal N of endogenous origin, which includes undigested duodenal EN (free and incorporated into bacteria) plus indigested intestinal EN, represented 31% of total N excreted, similar to values reported by Larsen et al.

(2001) and Mason (1969) using fecal water-soluble fraction as representative of endogenous origin. Based on our model description, 72% of the EN loss in feces originated from undigested EN secretions from the forestomach, similar to previous observations with the same model (Ouellet et al., 2002).

Absorption of N from the forestomach, representing mainly ammonia, tended to increase ($P < 0.10$) for cows fed silages and represented 42, 56, and 53% of N intake

Table 3. Secretions, absorptions, and digestibilities of N across sections of the digestive tract estimated using the gut mucosa (mucosa) or the average enrichment of the gut mucosa and milk casein (combined) as representative of the endogenous secretions in cows fed hay, formic acid-treated silage (formic), or inoculated silage (inoc)^{1,2}

Parameter	Mucosa			Combined			SEM	Contrast	
	Hay	Formic	Inoc	Hay	Formic	Inoc		Hay vs. formic and inoc	Formic vs. inoc
Secretions, g of N/d									
Urea N into forestomach	52	59	60	44	52	52	8 (7)	NS ³ (NS)	NS (NS)
Endogenous N into forestomach	142	143	147	75	73	72	9 (5)	NS (NS)	NS (NS)
Endogenous N into intestine	40	34	42	24	21	24	13 (7)	NS (NS)	NS (NS)
Absorption, g of N/d									
Forestomach	145	228	213	69	151	129	27 (22)	0.10 (0.06)	NS (NS)
Intestine	313	288	316	299	275	299	36 (33)	NS (0.10)	NS (0.03)
Real digestibility, %									
Forestomach	26	39	34	14	30	23	4 (4)	0.09 (0.06)	NS (NS)
Intestine	71	70	70	70	69	69	1 (1)	NS (NS)	NS (NS)
Whole tract	78	82	80	74	78	76	1 (1)	NS (0.06)	NS (NS)

¹Least squares means and the highest SEM based on n = 3, 4, and 4 for hay, formic, and inoculated, respectively.

²Contrast and SEM are presented as unique value for mucosa and combined methods when identical; if different, combined value is presented in parentheses.

³NS = $P > 0.10$.

Table 4. Flows of N (g/d) at the ileocecal area estimated using the gut mucosa (mucosa) or the average enrichment of the gut mucosa and milk casein (combined) as representative of the endogenous secretions in cows fed hay, formic acid-treated silage (formic), or inoculated silage (inoc)^{1,2}

Parameter, g of N/d	Mucosa			Combined			SEM	Contrast	
	Hay	Formic	Inoc	Hay	Formic	Inoc		Hay vs. formic and inoc	Formic vs. inoc
Ileal digesta	115	149	151	115	149	151	26	0.001	NS ³
Undigested duodenal digesta ⁴	102	139	133	107	143	140	27 (26)	NS (0.09)	NS (NS)
Endogenous N from undigested secretions into intestine	13	10	18	8	6	11	7 (5)	NS (NS)	NS (NS)
Endogenous N from undigested secretions into forestomach	23	30	31	14	18	19	3 (2)	NS (NS)	NS (NS)
Total endogenous N in ileal digesta	37	40	49	23	24	30	8 (5)	NS (NS)	NS (NS)

¹Least squares means and the highest SEM based on n = 2, 2, and 2 for hay, formic, and inoculated, respectively.

²Contrast and SEM are presented as unique value for mucosa and combined when identical; if different combined values are presented in parenthesis.

³NS = $P > 0.10$.

⁴Undigested duodenal digesta = total ileal N – endogenous N from undigested secretions into intestine.

for hay, formic, and inoc, respectively (Table 3). These proportions are similar to estimates obtained in sheep fed diets that exceeded N requirement for microbial protein synthesis (Hettiarachchi et al., 1999). The current results also agree with those of Parker et al. (1995), who reported that 50% of N intake enters the rumen irreversible loss of ammonia pool. The real digestibility of N in the forestomach tended to be higher ($P = 0.09$) for cows fed silages (37%) than for those fed hay (26%), reflecting the rapidly degradable N content of silages compared with hay (Martineau et al., 2007).

Endogenous N flows at the ileum could be only estimated for the 2 cows with an ileal cannula. Total N flow at the ileum was higher ($P < 0.001$) when cows were fed the silages rather than hay (Table 4). For the cows fed inoc, this is probably because of the higher return of urea to the gastrointestinal tract. The reason is less clear for cows fed formic, although undigested feed at the duodenum was numerically greater and N absorption between the duodenum and the feces was numerically lower. The ileal EN flow was unaffected by treatment and represented, on average, 31% of ileal N flow. This value yields an average true small intestinal N digestibility for the combination of undigested feed, bacteria, and EN of 69%, similar to the 65 to 70% reported by Larsen et al. (2001) using 2 different approaches (the difference method and the AA profile method).

Together, these results illustrate that forage type can alter both free EN and total EN at the duodenum. In addition, contribution of EN to bacteria N can be more important than urea utilization. Indeed, the EN incorporated into bacteria is of similar magnitude to free EN flow at the duodenum. Approximately 30% of fecal N was as EN, of which 70% originates from the forestomach. The limited data from ileal EN flux sup-

port the conclusions based on fecal samples. Overall, these data clearly indicate the importance of including EN at the duodenum, both as free and incorporated into bacteria, to estimate with better accuracy the true net N supply to cows.

Limitations of Current Methodologies and Implications for Predictive Models

Precursor Pool. The uncertainty of the correct precursor pool(s) that represent the site of EN production is a major limitation to isotope-based techniques. Pancreatic tissue, gastric or intestinal mucosa, the TCA-soluble fraction of portal vein plasma (Hess et al., 1998), mucins (Lien et al., 1997; Leterme et al., 1998), or other export proteins directly linked to EN, such as pancreatic or bile juice (Souffrant et al., 1993), have been proposed.

In the current experiment, the IE of biopsies from the rumen and duodenal mucosa and milk casein differed ($P < 0.05$) and averaged 0.3944, 0.4013, and 0.4327 $\pm$ 0.0023 atom percent, respectively. These values are similar to those reported earlier (Ouellet et al., 2002). Combining the IE of mucosa and milk casein reduced the variation of EN estimations but affected only a few treatment differences compared with the use of mucosal IE as the precursor. Therefore, qualitative treatment responses in EN were independent of precursor selection, although the absolute values were higher for the mucosal method. For example, the average secretion of EN into the forestomach and the intestine were lowered by 41 and 49%, respectively (Table 2), for the combined method compared with the mucosal method. Similarly, average absorption was reduced by 41 and 5% at the forestomach and postduodenal sites, respectively, when comparing the combined method with the

mucosa method (Table 3), whereas real digestibility in the forestomach was also reduced by 46% for hay and 26% for silages. These examples emphasize the effect of precursor pool selection, especially when the absolute contribution of EN is the objective. Regrettably, an ideal sample, technically easy to obtain and representative of the precursor pool, does not currently exist. However, because of the concordance observed between values published in the literature and the present study, the rumen mucosa seems appropriate to evaluate EN, at least at the duodenum. Given the low number of results accumulated until now, the use of the mucosal tissue in the calculation of EN flow at the ileum remains to be evaluated.

Implications for Predictive Models. This project is part of a wider initiative to develop quantitative estimation of EN flows in dairy cows. This necessitates assessment of the current estimates against reported values obtained by other approaches to assess the validity of the model.

Of the total duodenal N, free EN plus EN incorporated in bacterial N contributed 26, 23, and 24% for hay, formic, and inoc, respectively (Table 2). These values are higher than the 15% reported previously using the same approaches (Ouellet et al., 2002) and can be explained by a larger contribution of EN to bacteria biomass (58% in the present project vs. 48% in Ouellet et al., 2002). Based on a simulated conventional dairy ration, Marini et al. (2008) estimated that EN contributed 35% to the duodenal flow, but this value included the contribution of urea to bacteria protein synthesis. Inclusion of this fraction within the current study raises the EN contribution to 34% of duodenal flow (mucosa method). With a DMI of 17.5 kg/d, the measured EN at the duodenum corresponded to 5.5 g of N/kg of DMI, almost 3-fold the value estimated with the NRC (2001) model. Interestingly, the free EN measured (2.3 g of N/kg of DMI) is similar to the 1.9 g of N/kg of DMI estimated for EN by the NRC (2001) model. Our model distinguishes between the sources of N used for microbial growth and shows EN as an important source for bacterial N; as such, this affects AA requirements and, if unaccounted, could lead to an overestimate of true supply.

The EN fecal losses provide a direct estimation of metabolic fecal protein (MFP). The NRC (2001) model estimates MP requirement for MFP as $\text{DMI} \times 30 \text{ g/d}$ (Swanson, 1977) minus estimated undigested bacteria. For cows consuming 17.5 kg of DM/d with an average duodenal flow of bacteria of 246 g of N/d, MP requirement for MFP according to NRC (2001) equals 393 g of MP/d. In the current study, fecal EN losses, calculated using either the mucosa or combined method, yields

40 and 24 g/d, respectively. Even the highest value that equates to 250 g of protein/d is lower than the estimated 393 g of MP/d (NRC, 2001). Nonetheless, as stated by Swanson (1982), “whichever portion of the fecal N is designated MFP, it is converted to maintenance requirement for protein only when modified by appropriate factors for utilization efficiency of feed protein.” When an average efficiency factor of 0.67 (NRC, 2001) is applied, the estimated MFP are raised to 373 g of MP/d. In a meta-analysis using the Lucas principle, Marini et al. (2008) obtained an MFP of 3.96 g of N/kg of DM equivalent to 68 g of fecal EN/d if applied to our cows, also higher than our maximal estimation of 40 g/d. These overestimations compared with value obtained in the present study could be related to the fact that both the NRC (2001) and Marini et al. (2008) models include urea N contribution to MFP.

CONCLUSIONS

The results of this study demonstrate that feeding hay compared with silages increased free EN flow at the duodenum but had no effect on ileal or fecal EN flows. This study showed the important contribution of EN to bacteria N flowing at the duodenum as well as free endogenous and urea input to duodenal flux. The model also indicated that approximately one-third of the total N flowing out of the ileal or fecal segment was of EN origin. In the current model, the MFP do not include urea, which differs from the NRC (2001) model. Although the MFP losses estimated using the NRC (2001) model are close to estimates obtained with the current model, when the latter was adjusted for an efficiency factor, the effect of diet composition, variation of N efficiency, and detail about AA composition would be better investigated using the ^{15}N dilution technique.

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