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Use of Nitrate-nitrogen as a Sole Dietary Nitrogen Source to Inhibit Ruminal Methanogenesis and to Improve Microbial Nitrogen Synthesis *In vitro*

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ABSTRACT : An *in vitro* study was conducted to determine the effect of nitrate-nitrogen used as a sole dietary nitrogen source on ruminal fermentation characteristics and microbial nitrogen (MN) synthesis. Three treatment diets were formulated with different nitrogen sources to contain 13% CP and termed i) nitrate-N diet (NND), ii) urea-N diet (UND), used as negative control, and iii) tryptone-N diet (TND), used as positive control. The results of 24-h incubations showed that nitrate-N disappeared to background concentrations and was not detectable in microbial cells. The NND treatment decreased net CH_4 production, but also decreased net CO_2 production and increased net H_2 production. Total VFA concentration was lower ($p<0.05$) for NND than TND. Suppression of CO_2 production and total VFA concentration may be linked to increased concentration of H_2. The MN synthesis was greater ($p<0.001$) for NND than UND or TND (5.74 vs. 3.31 or 3.34 mg/40 ml, respectively). Nitrate addition diminished methane production as expected, but also increased MN synthesis. (**Key Words :** Nitrate Nitrogen, Ruminal Methanogenesis, Microbial Nitrogen Synthesis, Gas Production, Rumen Fermentation)

INTRODUCTION

To protect the environment and improve the energetic efficiency of ruminant animals, it would be beneficial to decrease methane production in the rumen. Nitrate has been shown to suppress methane production by rumen microbes (Jones, 1972; Takahashi and Young, 1991). This is because the electrochemical reduction of 1 mol nitrate to ammonia consumes 8 mol of electrons, e.g., hydrogen (Richardson and Watmough, 1999). The dependence of methanogenesis and nitrate reduction on electron sources is described by the following two stoichiometries:

$4H_2+HCO_3^-+H^+ \rightarrow CH_4+3H_2O$; $\Delta G_o' = -175$ kJ/reaction (Conrad and Wetter, 1990)

$NO_3^-+2H^++4H_2 \rightarrow NH_4^++3H_2O$; $\Delta G_o' = -598$ kJ/reaction (Allison and Reddy, 1984)

Since methane production is less when nitrate is added, it is presumed that nitrate- and nitrite-reducing microbes successfully compete with methanogenic microbes for hydrogen in the rumen (Allison and Reddy, 1984; Iwamoto et al., 1999; 2001).

Microbial growth in the rumen is a function of the amount of energy derived from ruminal fermentation processes. Energy is seldom abundant for growth of rumen microorganisms (Russell and Wallace, 1997). Reductive processes that allow for greater conservation of energy for growth should be advantageous to organisms which conduct these processes. Thermodynamics predicts that nitrate reduction should be energetically more beneficial than methanogenesis and therefore support more microbial growth. Allison and Reddy (1984) tested the effect of nitrate on cell yield by rumen microbes in continuous culture but considered their results to be inconclusive. We are not aware of other tests of this hypothesis with rumen microbes.

The objective of the present study was to investigate the effect of nitrate-nitrogen as a sole nitrogen source on ruminal fermentation characteristics and microbial nitrogen (MN) synthesis using an *in vitro* gas production method.

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Table 1. Ingredients and chemical composition of the experimental treatments

Item	Treatment		
	NND[1]	UND	TND
Ingredients			
Soluble starch (% DM)	57.4	65.5	53.3
Avicel (% DM)	30.0	30.0	30.0
Tryptone (% DM)	-	-	16.7
Urea (% DM)	-	4.5	-
Sodium nitrate (% DM)	12.6	-	-
Chemical composition[2]			
Crude protein (% DM)	13.1	13.2	12.7

[1] NND = Nitrate-N diet; UND = Urea-N diet; TND = Tryptone-N diet.
[2] Actual determination.

MATERIALS AND METHODS

In vitro substrates

Three substrate diets were formulated with urea (U4128, Sigma-Aldrich Chemical Co., St. Louis, MO), tryptone (Oxoid Ltd., Basingstoke, Hampshire, UK), or sodium nitrate (S5506, Sigma-Aldrich) as sole N sources, and with various amounts of soluble starch (S4251, Sigma-Aldrich) and Avicel (GH-9471, Fluka, Chemie GmbH) to balance the dietary N content (13% CP). Tryptone is a pancreatic digest of casein and a source of amino acids and peptides. The ingredient and chemical compositions are presented in Table 1.

In vitro incubations

All procedures involving animals were conducted under approval of the China Agricultural University Institutional Animal Care and Use Committee. Ruminal fluid was obtained from three cannulated Simmental×Luxi steers fed diets (8 kg DM/d), consisting of 60% roughage (corn stover cubes and alfalfa hay pellets) and 40% mixed concentrate (ground corn, soybean meal and soyhulls) twice daily. No nitrate was added to the diet of the donor steers. The chemical composition (on DM basis) of the diet was 13.2% CP, 39.6% NDF, 23.5% ADF, 1.14% Ca and 0.68% P. There was no detectable nitrate in the concentrate mix. Based on the nitrate content of alfalfa (1.11 mg/g DM) and corn stover (0.56 mg/g DM) and their diet proportions (25% and 35%, respectively), the nitrate intake of the donor steers was less than 4 g/head/d.

Rumen fluid (1 L) was collected from each steer, filtered through four layers of cheesecloth, and then pooled. The rumen fluid filtrates were poured into an anaerobic buffer solution in 8-L flasks under a constant flow of O_2-free CO_2 and homogenized in a blender under CO_2 for 2 min. Buffer solution was prepared in the ratio of 400 ml distilled water, 0.1 ml trace element solution A, 200 ml buffer solution B, 200 ml macro-element solution C, 1 ml resazurin solution (0.1% w/v) and 40 ml reductant solution. Trace element solution A contained $CaCl_2 \cdot 2H_2O$, 13.2 g; $MnCl_2 \cdot 4H_2O$, 10.0 g; $CoCl_2 \cdot 6H_2O$, 1.0 g and $FeCl_3 \cdot 6H_2O$, 8.0 g, which were dissolved in 100 ml distilled water. Buffer solution B was $NaHCO_3$, 35 g, dissolved in 1,000 ml distilled water. Macro-element solution C was Na_2HPO_4 5.7 g; KH_2PO_4, 6.2 g and $MgSO_4 \cdot 7H_2O$, 0.6 g, dissolved in 1,000 ml distilled water. Reductant solution consisted of 1 *M* NaOH, 4 ml; $Na_2S \cdot 9H_2O$, 0.625 g, and 95 ml distilled water. Forty milliliters of the mixed culture medium (ratio of ruminal fluid:buffer = 1:2) were pipetted with an automatic pump into replicate glass syringes (HFT000025, Häberle Maschinenfabrik GmbH, Germany) loaded with 0.2 g DM of substrate diets and pre-warmed to 39°C. The syringes were then incubated in a shaking water bath at 39 °C. Blank syringes containing only mixed culture medium with no substrate addition were simultaneously incubated. There were nine replicate syringes for each substrate diet, three for measurement of gas production over 72 h after the procedure of Menke et al. (1979), three for measurement of 24-h gas composition and three for 24-h sampling of soluble analytes and microbial nitrogen (MN). The piston position on the syringe was noted at several incubation times for determination of the gas production rate. Gas production measurements (ml/0.2 g DM) were taken after 0, 2, 4, 6, 8, 12, 16, 24, 32, 40, 48, 56, 64 and 72 h of incubation and the results corrected for the blank were fitted to the one-component exponential model (logistic curves) of Schofield (2000) with a minor modification: $Y = A \times (1-\exp(-C \times (t-Lag)))$, where Y is *in vitro* gas volume (ml/0.2 g DM) at time t, A is the asymptotic potential gas volume (ml/0.2 g DM), C is the specific rate of gas production (h^{-1}), and Lag is the time elapsed (h) before gas production started. The parameter estimates for A, C, and Lag for each N source were calculated using a non-linear regression procedure (SAS, 2003). Each experiment was repeated in triplicate on different days.

Sampling and analyses

Gas composition : After 24 h of incubation, 0.5 ml headspace gas was taken from each syringe using a 1-ml glass syringe, and then analyzed for composition with an Agilent 6890N gas chromatograph fitted with a TDX-01 packed column, 1 m×3 mm×2 mm (Benfen Ruili Instrument Co., Beijing, China). The carrier gas was argon with a flow rate of 30 ml/min. Temperatures of the injector port, column and thermal conductivity detector port were 120°C, 100°C and 160°C, respectively. Bridge current was 94 mA. Determination of gas composition was accomplished by reference to a standard gas consisting of 7.97% nitrogen, 65.3% carbon dioxide, 1.77% hydrogen and the balance as methane.

NH_3-N, NO_3^-, NO_2^- and VFA : Gas profile and soluble

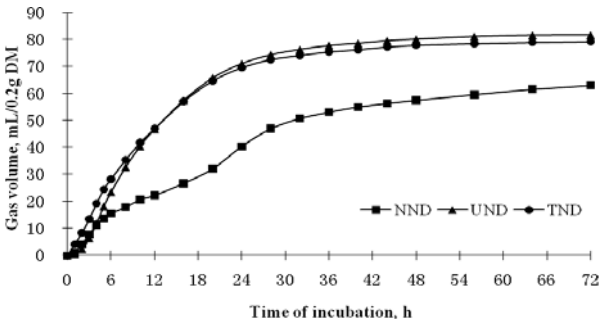

Figure 1. Effect of dietary nitrogen sources on kinetics of *in vitro* net gas production during 72 h incubation.

analytes were sampled at the end of 24-h incubation, which was assumed to be before incubation conditions became less rumen-like and prior to secondary fermentation of microbial biomass due to substrate depletion. Contents of the syringes were transferred to pre-weighed centrifuge tubes. The pH of culture fluid was measured immediately with a pH meter equipped with a glass electrode (Model PHS-3C, Shanghai Leici Scientific Instrument Co., Ltd., China). Three milliliters of culture supernatant were collected after centrifuging for 15 min (5,400×g) for determination of ammonia-nitrogen (NH_3-N), VFA, NO_3^- and NO_2^-. The remaining syringe contents were prepared for determination of MN. Ammonia-N concentration was measured by the procedure of Broderick and Kang (1980) using a spectrophotometer (UV-VIS 8500, Shanghai Tianmei Scientific Instrument Co., Ltd., China). One milliliter of the supernatant was acidified with 25% metaphosphoric acid, and then frozen at -20°C. Upon thawing, the acidified supernatant samples were centrifuged for 15 min at 10,000×g and analyzed for VFA using an Agilent 6890N gas chromatograph equipped with a 30-m×0.32-mm i.d. fused Supelcowax TM-10 capillary column (Supelco, Inc., Bellefonte, PA, USA) in split mode (ratio 1:100) with a flame ionization detector. Sample injection volume was 0.6 μl. The carrier gas was helium with a flow rate of 50 ml/min and 2-ethylbutyric acid was used as an internal standard. The injector port temperature was 280°C, FID detector temperature was 280°C, and the oven temperature was held at 145°C. Net VFA production was calculated as total VFA production in treatment tubes minus total VFA production of blank tubes. Nitrate and nitrite concentrations in the culture fluid were determined with an ion chromatograph (Model Dionex-2500, Dionex Co., Ltd, USA) using an Ionpac AS11-HC 2-mm analytical column, and the eluent was 30 m*M* NaOH at a flow rate of 0.38 ml/min. Standard solutions were prepared with nitrate ion standard solution (72544, Fluka, Chemie GmbH) and nitrite ion standard solution (72586, Fluka, Chemie GmbH). Net nitrate and nitrite concentration was calculated as the concentration of nitrate and nitrite in treatment tubes minus nitrate and nitrite concentration of blank tubes. To assess whether NO_3^- and NO_2^- accumulated in the microbial cells, cell pellets were suspended in saline solution and then an ultrasonic probe was used to disrupt the cells.

Microbial nitrogen determination : After supernatant sampling for determination of NH_3-N, VFA, NO_3^- and NO_2^-, 37% formaldehyde solution was added to the remaining syringe contents, at a fluid proportion of 2.5%, and the syringe contents were resuspended. After stirring for 2 min, the contents were centrifuged at 10,000×g for 20 min at 4°C and the supernatant was discarded. The pellets were suspended in 30 ml physiological saline, centrifuged as before and supernatant discarded. This washing procedure was repeated a second time. The pellets in pre-weighed centrifuge tubes were completely lyophilized for 3 d (Model 44C2-A, Beijing Boyikang Instrument Co., Ltd, China) and weighed. Finally, the N content of pellet residues in each tube was determined using a Nitrogen Analyzer (Model Rapid N III, Elementar, Germany) based on the combustion method (AOAC, 990.03, 1990). Because starch and Avicel are nitrogen-free and the three sources of N (urea, tryptone, and sodium nitrate) are completely soluble, any undegraded dietary N was washed out of the pellets. Therefore, pellet residual N was assumed to be mainly from rumen microbial synthesis with trace amounts from undegraded feed particles associated with the rumen fluid inoculum. Net MN synthesis was calculated as pellet residual N content of treatment tubes minus pellet residual N content of blank tubes. Total N initially present in each tube was calculated as the sum of added substrate N, pellet residual N in blank 24-h incubations and NH_3-N in blank 24-h incubations.

Statistical analysis

Data for culture fluid chemical composition, gas production parameters, gas composition, VFA and ammonia-N were subjected to analysis of variance in a randomized complete block design using the general linear model of SAS (SAS Institute, 2003). Source of dietary N was treated as a main effect, and experimental replicate as block. Because the block effect was not significant ($p > 0.05$), data were then pooled and analyzed using one-way analysis of variance. Multiple comparison tests used Duncan's multiple-range test (Snedecor and Cochran, 1980).

RESULTS

Effect of dietary nitrogen sources on gas profile and rumen fermentation acids *in vitro*

Compared with urea and tryptone N sources, the nitrate-N diet (NND) exhibited a different gas production pattern (Figure 1). With NND, there was a suppression of 72-h gas

Table 2. Effect of dietary nitrogen sources on net gas production and gas composition after 24-h incubation *in vitro*

Item	Treatment			SEM[2]	p-value
	NND[1]	UND	TND		
Net gas production (ml/0.2 g DM)	40.4[b]	71.1[a]	69.6[a]	0.87	<0.0001
Gas proportion (%)					
CO_2	84.3[a]	79.6[b]	79.6[b]	0.67	<0.0001
CH_4	7.5[b]	17.2[a]	16.8[a]	0.33	0.004
N_2	8.1[a]	3.9[b]	3.8[b]	0.68	0.007
H_2	0.116[a]	<0.001[b]	0.014[b]	0.01	<0.0001
Gas volume (ml)					
CO_2	34.1[b]	56.6[a]	55.4[a]	0.74	<0.0001
CH_4	3.05[c]	12.2[a]	11.7[b]	0.14	<0.0001
N_2	3.3	2.8	2.6	0.21	0.134
H_2	0.046[a]	<0.001[b]	0.010[b]	<0.01	<0.0001

[a, b, c] Within a row, means without a common superscript letter differ (p<0.05).
[1] NND = Nitrate-N diet; UND = Urea-N diet; TND = Tryptone-N diet. [2] SEM = Standard error of the mean.

volume (p<0.05) and gas production rate (p<0.05). The gas production lag time of NND was not different (p>0.05) from that of the tryptone-N diet (TND), but shorter (p<0.05) than that of the urea-N diet (UND). Rate of gas production was notably slow for NND between 6 and 24 h. When the gas volume data from 24 h to 72 h were fitted to the one-component exponential model of Schofield (2000), there was no difference (p = 0.358) in specific rate of gas production due to treatment.

Consistent with 72-h incubations (Figure 1), net gas production at 24-h was lower (p<0.05) for NND than for the UND and TND treatments (Table 2). A lower percentage of CH_4 (p<0.05) and higher percentages of CO_2, N_2 and H_2 (p<0.05) were observed for NND than UND and TND, respectively. For clarity in assessment of treatment effects, net gas production and composition data were used to calculate volumes of the component headspace gases (Table 2). The NND treatment diminished (p<0.05) CO_2 and CH_4 volumes and led to higher (p<0.05) net H_2 accumulation. There was no treatment effect (p>0.05) on volume of N_2, as expected (Table 2).

There was a higher (p<0.05) pH value in NND compared with the other two N sources (Table 3). Total VFA concentration of NND was not different from that of UND, but lower (p<0.05) than that of TND (Table 3). For VFA molar proportions, NND produced higher acetate and lower butyrate molar proportions (p<0.05) than UND and TND (Table 3). Dietary N sources had little influence (p>0.05) on the molar proportions of propionate, isobutyrate, isovalerate and valerate (Table 3).

Effect of nitrogen sources on NO_3^-, NO_2^-, NH_3-N, and MN synthesis

Following 24-h of incubation, no differences (p>0.05) were found in concentrations of NO_3^- and NO_2^- in culture supernatants from the three dietary N treatments (Table 4). NO_3^- and NO_2^- were not detected within the microbial cells from the three diets (data not shown), which is presumed to indicate that all NO_3^- added to NND was reduced to NH_3-N by rumen microorganisms. NH_3-N concentration was lower (p<0.05) in NND than in UND and TND. Dietary nitrogen source affected MN synthesis (p<0.05). Synthesis of MN was greater (p<0.05) for NND than for UND and TND. After 24-h incubations, NND had a higher (p<0.05) percentage of MN and a lower (p<0.05) percentage of NH_3-N in total nitrogen than UND and TND (Figure 2).

Table 3. Effect of dietary nitrogen sources on *in vitro* rumen fermentation characteristics after 24-h incubation

Item	Treatment			SEM[2]	p-value
	NND[1]	UND	TND		
pH	6.67[a]	6.62[b]	6.61[b]	0.01	0.010
Net TVFA (mM/L)	32.0[b]	37.2[b]	45.9[a]	3.31	0.053
VFA molar proportion (*M* %)					
Acetate	67.5[a]	61.7[b]	63.7[b]	0.90	0.002
Propionate	21.6	22.6	20.8	0.61	0.161
Isobutyrate	1.40	1.53	1.45	0.15	0.163
Butyrate	5.47[b]	9.82[a]	9.58[a]	0.60	0.001
Isovalerate	2.78	2.93	3.04	0.08	0.107
Valerate	1.33	1.40	1.37	0.12	0.820

[a, b, c] Within a row, means without a common superscript letter differ (p<0.05).
[1] NND = Nitrate-N diet; UND = Urea-N diet; TND = Tryptone-N diet. [2] SEM = Standard error of the mean

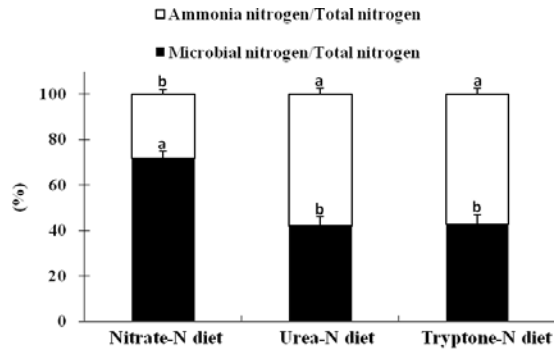

Figure 2. Distribution of ammonia- and microbial-nitrogen in total N as affected by three dietary N sources after 24-h incubation *in vitro*. Error bars are SEM.

DISCUSSION

Nitrate and nitrite disappeared from the culture fluid of NND by 24 h of incubation (Table 4). These compounds did not accumulate in microbial cells. The disappearance of nitrate and nitrite is consistent with the presence of nitrate- and nitrite-reducing activity in rumen contents of sheep prior to nitrate addition (Allison and Reddy, 1984). In addition, Kaspar and Tiedje (1981) concluded that denitrification in the rumen was not quantitatively important. Experiments with ^{15}N-labeled nitrate and nitrite resulted in ^{15}N-NH_3, though possibly 0.3% of nitrite-N appeared as nitrous oxide-N.

The concentration of added nitrate in the NND incubations was 7.4 mM. Complete disappearance of this concentration within 24 h is consistent with the disappearance of nitrate and nitrite from rumen contents of a non-nitrate-adapted goat (Iwamoto et al., 2002).

The NND treatment resulted in less CO_2 and methane production by 24 h (Table 2). The gas volume results in Figure 1 indicate that the suppression of gas production rate was transient and concurrent with the disappearance of nitrate during h 0 to 24 of incubation. Suppression of methanogenesis by dietary nitrate has been reported previously with *in vitro* (Jones, 1972) and *in vivo* (Takahashi and Young, 1991) conditions. Allison and Reddy (1984) also mentioned that inhibition of methanogenesis was more pronounced with microbial populations from a NO_3^- adapted animal.

In anaerobic ecosystems, two hypotheses have been invoked to explain this suppression. One hypothesis is that there is superior interspecies transfer of hydrogen for reduction of nitrate to ammonia compared with reduction of CO_2 to CH_4, i.e., competition for H_2 (Cord-Ruwisch et al., 1988). The second hypothesis is that N-oxide intermediates, such as nitrous oxide, suppress methanogenesis, evidence for which was provided by KlÜber and Conrad (1998) using anoxic slurries of Italian rice soil. Tugtas and Pavlostathis (2007) provided corroborating evidence from their experiments with mesophilic, anaerobic digester cultures. Different methanogenic species show different sensitivity towards each N-oxide (Clarens et al., 1998; KlÜber and Conrad, 1998). Methanogenesis resumed after disappearance of the N-oxides. The relative importance of these two hypotheses in the context of ruminal methane suppression by nitrate is not known.

The VFA molar proportions (Table 3) revealed elevated acetate and decreased butyrate, by the NND treatment. Farra and Satter (1971) also observed increased acetate and decreased butyrate molar proportions *in vivo* due to dietary nitrate supplementation. Furthermore, Farra and Satter (1971) noted the nitrate decreased molar proportion of propionate. These alterations in VFA profile may be indicative of greater reducing equivalent release (Russell and Wallace, 1997) by the fermentative population due to nitrate reduction.

Although there was a decrease in N_2 proportion, the volumetric contribution of N_2 was not affected by treatment (Table 2). This result is consistent with the conclusion of Kaspar and Tiedje (1981). However, Kaspar and Tiedje (1981) mentioned that it is unknown whether denitrification might be more successful under high nitrate availability. In this trial with sodium nitrate as 12.6% of substrate DM and as the sole nitrogen source, there was no evidence for denitrification via *in vitro* production of N_2.

The experiments reported here were intended to evaluate whether nitrate supplementation affects microbial cell synthesis. The N from NND resulted in a lower proportion of total N present as NH_3-N (Figure 2) and lower NH_3-N concentration (Table 4) after 24 h due to greater synthesis of MN from NH_3-N. The advantage of NND over

Table 4. Effect of dietary nitrogen source on the net concentration of NO_3^-, NO_2^-, NH_3-N, and MN in 40 ml fermentation fluid after 24-h incubation *in vitro*

Item	Treatment			SEM	p-value
	NND[1]	UND	TND		
NO_3^- (mg/40 ml)	0.11	-0.08	0.35	0.28	0.7239
NO_2^- (mg/40 ml)	-0.04	-0.03	-0.12	0.08	0.6878
NH_3-N (mg/40 ml)	2.34[b]	4.71[a]	4.75[a]	0.11	<0.0001
MN (mg/40 ml)	5.74[a]	3.31[b]	3.34[b]	0.13	<0.0001

[a, b, c] Within a row, means without a common superscript letter differ ($p<0.05$).
[1] NND = Nitrate-N diet; UND = Urea-N diet; TND = Tryptone-N diet. [2] SEM = Standard error of the mean.

UND in MN occurred even though NND had a lower proportion of organic matter due to lower soluble starch content. Allison and Reddy (1984) hypothesized that, with NO_3^- as N source, there could be an increase in cell yield which might reflect an increase in ATP yield through the functioning of dissimilatory NO_3^- reduction. Nitrate was able to serve as an electron acceptor for *Clostridium perfringens*, permitting increased growth yields over those obtained in its absence (Hasan and Hall, 1975), and the effect of NO_3^- on MN synthesis is consistent with thermodynamic principles embodied in the electron tower concept (Madigan et al., 1997). There was no difference ($p<0.05$) between UND and TND for 24-h gas production or MN synthesis, thus indicating no effect of amino-N (TND) on microbial fermentation or growth rate.

Nitrate reduction to ammonium is more energetically favorable than HCO_3^- reduction to CH_4. The standard redox potential (Thauer et al., 1977) for NO_3^- reduction to NH_4^+ is more electropositive ($E_0' = 0.37V$) than that for HCO_3^- reduction to CH_4 ($E_0' = -0.24$ V). Nitrate reduction to NH_4^+ releases more energy (101.1 kcal/reaction) than HCO_3^- reduction to CH_4 under standard conditions ($\Delta G_o'$ at 25°C and pH = 7.0). In the rumen environment (39°C, pH = 6.8) and considering the concentrations of reactants and products, the free-energy advantage (ΔG) of nitrate reduction is estimated to be 85 kcal/reaction, as follows. According to Conrad and Wetter (1990) and Henry's law, ΔG for the reaction of HCO_3^- to CH_4 is -18.5 kcal/reaction ($p(H_2) = 137.7$ Pa (Hungate, 1967; Smolenski and Robinson, 1988); $p(CH_4) = 3.1\times10^4$ Pa (Hungate, 1967; Czerkawski and Breckenridge 1971); $C(HCO_3^-) = 3.5\times10^{-2}$ M (Turner and Hodgetts, 1955; Counotte et al., 1979)) and ΔG for the reaction of NO_3^- to NH_4^+ is -103.5 kcal/reaction ($p(H_2) = 137.7$ Pa; $C(NH_3\text{-}N) = 1.01\times10^{-2}$ M (Wohlt et al., 1976); $C(NO_3^-) = 1.5\times10^{-4}$ M (Table 4, NO_3^- concentration of NND)). Assuming that the thermodynamic advantage is 85 kcal/reaction, an energy equivalence of 1 mol ATP is 10.5 kcal at pH 7 (Thauer et al., 1977), bacterial growth efficiency is 11.6 g cells per mole of ATP (Isaacson et al., 1975), and cellular crude protein content is 47% (Merry and McAllan, 1983), the addition of 25.5±0.78 mg sodium nitrate in the NND treatment would result in an additional 2.10±0.06 mg MN. This calculated advantage is consistent with finding an additional 2.43 mg MN and 2.40 mg MN when comparing NND to UND and TND, respectively (Table 4). Whether increased MN synthesis is due to a nitrate-induced proliferation of nitrate-reducing species, increased growth of the pre-existing fermentative organisms, proliferation of pre-existing subliminal microbial populations or a combination of these factors would be an interesting microbial community analysis.

The NND treatment resulted in less and slower total net gas production (Figure 1 and Table 2). Comparing the TND and UND treatments, NND had 30.7 ml less 24-h net gas production (Table 2). This is expected to the extent that there was less CH_4 (9.15 ml) produced, but then the implication is that this should have resulted in more CO_2 production by the NND treatment. In fact, there was less CO_2 (22.5 ml) production by NND than UND. This result is corroborated by the lower total VFA concentration for NND which seems to be consistent with the elevated pH of NND incubations (Table 3). Of course, increased MN synthesis should result in greater carbon assimilation. The sharply decreased gas production within 24 h and partial recovery thereafter (Figure 1) suggests that there may be a transitory inhibition by nitrate. Extent of DM digestion was not determined in these experiments. It is pertinent that Marais (1988) found that rumen cellulolytic and amylolytic activities were inhibited by the addition of nitrate to the diet.

Hydrogen concentration was elevated due to NND (Table 2). This is unexpected given the results of Cord-Ruwisch et al. (1988), who reported threshold concentrations of H_2 to be two orders of magnitude lower when nitrate was the electron acceptor as opposed to CO_2. Conversely, Russell and Jeraci (1984) presumably inhibited hydrogenase activity in ruminal fermentative organisms via carbon monoxide introduction with the consequences that headspace H_2 was elevated and OM digestion was depressed. It will be important to determine whether nitrate as an alternative electron acceptor has an effect on DM digestibility in adapted ruminants.

Although abrupt introduction of nitrate into ruminant diets can result in methemoglobinemia (Emerick, 1988), dietary inclusion of up to 5% of average daily feed intake did not impair milk production when adequate time was allowed for cow adaptation to the diet (Farra and Satter, 1971). Similarly, no signs of toxicity were observed when mature sheep were adapted to a diet containing 4% KNO_3 (Caver and Pfander, 1974). Lichtenwalner et al. (1973) gradually adapted finishing beef steers to dietary potassium nitrate and reported that levels of 0, 1.0, or 2.0% in corn gluten meal- and soybean meal-based diets did not affect cattle growth performance.

IMPLICATIONS

Compared with tryptone and urea as dietary N sources, sodium nitrate addition led to lower CH_4 production, an increased proportion of acetate, greater rumen microbial protein synthesis and apparently some suppression of *in vitro* rumen fermentation as indicated by lower total VFA concentration and less CO_2 production. Headspace hydrogen concentration was increased in 24-h incubations which may be related to the suppressed microbial fermentation. Nitrate has potential value as a dietary nitrogen supplement for improving rumen microbial protein

synthesis and mitigating methane emissions, but the effects of this alternative electron acceptor on ruminal digestion should also be addressed.

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