JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Nitroethane and Monensin on Ruminal Fluid Fermentation Characteristics and Nitrocompound-Metabolizing Bacterial Populations

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Nitroethane is a potent inhibitor of ruminal CH_4 production, a digestive inefficiency resulting in the loss of 2−15% of a ruminant's gross energy intake and an important emission source of this greenhouse gas. To assess the effect of nitroethane on methanogenesis and characterize ruminal adaptation observed with low treatment doses to this inhibitor, ruminal microbes were cultured in vitro with supplements of water (controls), 4.5 and 9 mM nitroethane, and 0.09 mM monensin, with or without 9 mM nitroethane. All treatments decreased CH_4 production >78% compared to controls; however, differential effects of treatments were observed on CO_2, butyrate isobutyrate, and valerate production. Treatments did not affect H_2 accumulation or acetate and propionate production. Most probable numbers of nitrometabolizing bacteria were increased with 4.5 and 9 mM nitroethane compared to numbers recovered from controls or monensin-containing treatments, which may explain ruminal adaptation to lower nitroethane treatments.

KEYWORDS: Methane; nitroethane; monensin; nitrometabolizing bacteria

INTRODUCTION

Methane is a reduced end product of ruminal fermentation processes and contributes to the rumen microbial ecology by maintaining a low partial pressure of H_2, enabling reduced nucleotide reoxidation by hydrogenase activity rather than by alcohol or lactate dehydrogenases (*1*). However, CH_4 formation also represents a loss of 2−15% of the ruminant's gross energy intake (*2*), and its inhibition is directly related to improvements in feed efficiency. Also, there is the increasing concern that global climate is being changed due to the accumulation of greenhouse gases (*3, 4*) to which CH_4 is considered to be 21 times more effective than CO_2 at trapping heat in the atmosphere (*5*).

Numerous chemicals that directly inhibit methanogens or biochemical conversions involved in methanogenesis have been investigated for their potential to decrease ruminal methanogenesis. Whereas these caused significant decreases in CH_4 production, most also caused increased H_2 and decreased acetate accumulations along with compensatory increases in accumulations of reduced fermentation acids such as propionate or butyrate (*2, 6, 7*). Moreover, the CH_4-inhibiting effect of many of these chemical inhibitors was often transient because the microbial population was able to adapt to the ecological perturbations (*7*). Ionophores such as monensin indirectly decrease ruminal methanogenesis, but again the decreases have been transient and the rumen fermentation profile is changed to result in decreased accumulation of acetate and increased accumulation of propionate (*8, 9*). However, public health concerns that agricultural antibiotic use may lead to the development of antimicrobial resistance have resulted in restrictions on the use of ionophores in the European Union (*10*). Furthermore, an increase in demand for organically produced beef may ultimately restrict the use of ionophores in the United States (*11*).

Attempts to develop ruminal methanogenesis reduction strategies have also focused on providing alternative electron acceptors to redirect electron flow away from the reduction of CO_2 to CH_4 while conserving the energetic synergies associated with H_2-transfer reactions (*12, 13*). For instance, nitrate effectively consumes electrons at the expense of CH_4 production within

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10.1021/jf800756c CCC: $40.75
Published on Web 05/21/2008

rumen ecosystems; however, the toxicity of nitrite, an intermediate produced during microbial reduction of nitrate to ammonia, is a major detriment to its use in animals (*14, 15*). Organic acids, such as fumarate (*16*), aspartate and malate (*17*), or oxaloacetate (*18*), also serve as alternative electron acceptors capable of redirecting electron flow away from methanogenesis but these will likely have limited usage until either costs of their supplementation decrease or the value of greenhouse gas credits increase dramatically (*19*). The butyrate precursors, crotonoic acid and 3-butenoic acid, were found to be ineffective as alternative electron acceptors (*20*).

Several short-chain nitrocomponds have been shown to inhibit ruminal methanogenesis by as much as 90% in vitro (*21*). Oral administration of nitroethane to cattle or sheep resulted in dose-dependent decreases (>43%) in CH_4-producing activity with no observable adverse effects on intake or animal well being (*22, 23*). Mechanistically, the nitrocompounds nitroethane, 2-nitroethanol, and 2-nitropropanol have been shown to inhibit ruminal formate oxidation and H_2 oxidation, thereby depriving methanogens of their major reductant sources (*24*). Unlike most other chemical inhibitors of ruminal methanogenesis, however, reducing equivalents not consumed by the reduction of CO_2 to CH_4 were not redirected toward increased production of more reduced fermentation acids such as propionate as nitrocompound inhibition of methanogenesis had little effect on molar proportions of volatile fatty acids produced (*21–23*). However, evidence has shown that rates of nitrocompound metabolism increase with continued exposure to the nitrocompounds, which suggests an enrichment of nitrometabolizing microbes (*23*). Enrichment of nitrometabolizing bacterial populations could contribute positively to rumen function by consuming reducing equivalents away from the reduction of CO_2 to CH_4. However, enrichment could contribute negatively by depleting nitrocompound concentrations to levels no longer inhibitory to methanogenesis. The objectives of the present study were to assess the direct effect of nitroethane, as a chemical inhibitor, on methanogenesis and to characterize the ruminal adaptation to this inhibitor, which we hypothesize may occur via enrichment of a nitroethane-metabolizing bacterial population.

MATERIALS AND METHODS

Experimental Design. Mixed populations of ruminal microbes were cultured during consecutive batch culture (*25*) in 18 × 150 mm crimp-top culture tubes containing basal medium, 0.2 g of finely ground alfalfa, and supplements to achieve 4.5 mM (1×) nitroethane, 9 mM (2×) nitroethane, 0.09 mM monensin, or 9 mM nitroethane plus 0.09 mM monensin (2× nitroethane plus monensin). Untreated controls were cultured likewise. Treatments and controls were conducted in triplicate. Cultures were initially inoculated with 1 mL of the particle-free ruminal fluid within 30 min of collection. The ruminal fluid was obtained from a ruminal cannulated Holstein−Friesian steer grazing rye grass pasture and strained through a nylon paint strainer (*26*). The pH of the rumen fluid before inoculation into tubes was 6.72. Basal medium, which was prepared and distributed aseptically (9 mL/tube) using anaerobic technique (*27*), contained 40% clarified rumen fluid (*14*) and (mg/100 mL) K_2HPO_4, 22.5; KH_2PO_4, 22.5; $(NH_4)_2SO_4$, 45; NaCl, 45; $MgSO_4 \cdot 7H_2O$, 4.5; $CaCl_2$, 2.25; resazurin, 0.1; cysteine-HCl, 50; and Na_2CO_3, 400. Supplements were administered to each tube via additions of 0−0.6 mL of distilled water, 0.2 or 0.4 mL of 239 mM nitroethane, or 0.2 mL of 5 mM monensin (in ethanol) to achieve a final volume of 10.6 mL in each tube after inoculation. The stock solution of nitroethane was prepared via dilution of the sodium salt (*23*) with distilled water. After 24 h of incubation (39 °C) under an H_2/CO_2 (1:1) gas phase, 1 mL from each culture was used to inoculate the next series of anaerobic culture tubes, which contained fresh medium, alfalfa, and respective water or treatment supplements. A total of 16 series were conducted in this way, by sequential transfer of inoculum from the previous series of tubes to fresh medium. Because each resultant population had opportunity to respond independently upon each successive transfer, each was considered an independent experimental unit. Methane, H_2, and CO_2 were measured on series 1, 2, 3, 6, 10, 13, and 16. Ammonia, net VFA production, lactate, and NE concentration were measured on series 1, 2, 3, 6, and 10. Net VFA production was calculated as the difference between the start time (0 h) and the end time (24 h) for each incubation series.

Most probable numbers of nitrometabolizing bacteria were estimated using a three-tube Most Probable Number method (*28*) after the sixth series of the experiment. The culture medium was prepared by employing medium B (*14*) supplemented with 6 mM of 3-nitro-1-propionic acid. After 14 days of incubation (39 °C) under anaerobic conditions (H_2/CO_2; 1:1 gas phase), the remaining 3-nitro-1-propionic acid was quantified. All cultures considered to be positive for nitrocompound-reducing bacteria had metabolized >50% of the added 3-nitro-1-propionic acid. 3-Nitro-1-propionic acid was used as the test substrate because it is less volatile than nitroethane. Both compounds, as well as a variety of similar nitrocompounds, are at present known to be metabolized in appreciable amounts only by the ruminal bacterium *Denitrobacterium detoxificans* (*29*).

In a final experiment, 1 mL volumes of populations cultured consecutively over 16 batch cultures in their respective treatments were transferred as before to fresh medium containing added alfalfa but lacking additions of previous treatments. After 24 h of incubation (39 °C) under H_2/CO_2 (1:1), the headspace gas was sampled and analyzed to assess the potential for resurgence in CH_4 production upon removal of the inhibitory pressure exerted the previous 16 incubations.

Analytical Procedures. Concentrations of H_2, CH_4, and CO_2 were measured via gas chromatography from samples collected from the headspace of each tube (*30*). Amounts of CH_4 measured represent amounts produced as incubations were supplied with fresh H_2/CO_2 (1:1) at the beginning of each incubation. Amounts of CO_2 accumulated were calculated as the difference between amounts measured after 24 h of incubation and the amount supplied (35 μmol mL^{-1} of incubation fluid). Amounts of H_2 oxidized were calculated as the difference between that supplied (35 μmol mL^{-1} of incubation fluid) and that measured at the end of each incubation. Volatile fatty acids were determined from the fluid contents using gas chromatography (*31*). Concentrations of nitrocompounds and ammonia were determined colorimetrically (*23, 32*). Lactate was measured enzymatically (*33*). Estimates of amounts of lactate and ammonia produced and amounts of nitroethane reduced were adjusted to account for the 10% carry-over that occurred with each inoculum. Estimation of the fate of reductant during the consecutive batch cultures was estimated via calculation of hydrogen balance (*18*) modified to contain terms for H_2 equivelants produced from VFA, lactate, and ammonia and for H_2 equivalants incorporated within VFA and lactate and in amounts of nitroethane metabolized. Terms for formate and ethanol were removed from the equation as these products were not measured. Consumption of 3 μmol of H_2 by the presumed reduction of each micromole of nitroethane to ethanamine, the presumed product of nitroethane reduction, was also calculated and is based on the stoichiometry reported by Angermeier and Simon (*34*) for the reduction of 2-nitroethanol to 2-aminoethanol by *Clostridium pasteurianium*. Estimates of CH_4 and CO_2 were derived from the fermentation balance of Wolin (*35*), which uses stoichiometric and oxidation state relationships of measured amounts of acetate, propionate, and butyrate to predict amounts of CH_4 and CO_2 produced.

Statistical Analysis. Tests for main effects of treatment were conducted by assuming a completely randomized design. Effects of treatment within series were tested by multiple comparisons of least-squares means adjusted by Tukey to assess treatment differences ($P < 0.05$) on CH_4, CO_2, H_2, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and results from the H_2 balance; because differences were small, least-squares comparisons of means for ammonia and lactate were not presented. Orthogonal polynomial contrasts, specifically the linear and quadratic main effects adjusted for unevenly spaced sampling intervals, were also computed within treatment to assess the pattern during consecutive series and are presented where

Table 1. Effects of Nitroethane, Monensin, or Their Combined Treatment on Fermentation Parameters during Consecutive Batch Culture of Mixed Populations of Ruminal Microbes

	μmol of H_2 equiv mL^{-1} of incubation fluid after treatment with[a]						
parameter[b]	control	1× NE	2× NE	Mon	2× NE + Mon	*P* value	SEM[c]
CH_4 produced	8.5 g	0.9 hi	0.8 i	1.9 h	0.7 i	<0.0001	0.28
CO_2 accumulated	64.9 g	54.0 gh	48.4 h	39.9 hi	32.8 i	<0.0001	4.13
H_2 remaining	25.3	24.2	23.7	22.5	21.7	0.10	1.08
nitroethane reduced		4.1 i	8.0 g		7.7 h	<0.0001	0.05
ammonia produced	2.7	2.4	2.6	2.4	2.6	0.08	0.11
lactate produced	1.7 g	1.5 gh	1.4 h	1.6 gh	1.7 gh	0.0109	0.07
acetate produced	25.4	25.8	30.0	21.0	17.0	0.07	3.53
propionate produced	13.6	13.6	14.9	13.0	12.3	0.47	1.06
butyrate produced	4.8 g	5.6 g	6.1 g	1.7 h	1.9 h	<0.0001	0.55
C2/C3 ratio	1.9 g	1.7 g	1.9 g	1.6 gh	1.3 h	0.0174	0.20
isobutyrate produced	0.2 h	0.2 gh	0.3 g	0.1 h	0.2 h	0.0057	0.03
valerate produced	1.0 hi	1.6 gh	1.7 g	0.4 i	0.6 i	<0.0001	0.17
isovalerate produced	0.1	0.1	0.2	0.1	0.1	0.55	0.19
estimated CH_4[d]	11.8 gh	12.3 gh	14.4 g	8.1 gh	6.4 h	0.0067	1.77
estimated CO_2[d]	23.2 ghi	24.8 gh	27.9 g	16.4 hi	14.4 i	0.0016	2.78
H_2 equiv inputs[e]	101.6 gh	108.9 gh	121.4 g	81.8 h	74.9 h	0.0056	10.7
H_2 equiv incorporated[f]	78.5 g	62.1 h	77.4 g	39.3 i	58.5 h	<0.0001	3.9

[a] Treatments: control; 1× NE, 4.5 mM nitroethane; 2× NE, 9.0 mM nitroethane; Mon, 0.09 mM monensin; and 2× NE plus Mon, 9.0 mM nitroethane plus 0.09 mM monensin. Values within rows with unlike letters differ ($P < 0.05$). [b] CH_4, CO_2, and H_2 represent main effect means from measurements taken after series 1, 2, 3, 6, 10, 13, and 16; all other parameters represent main effect means from measuresments taken after series 1, 2, 3, 6, and 10. [c] SEM, standard area of the mean. [d] CH_4 and CO_2 estimates were derived from the fermentation balance of Wolin (*35*). [e] H_2 equiv inputs calculated as (2 equiv of acetate + 1 equiv of propionate + 4 equiv of butyrate + 3 equiv of valerate + 1 equiv of lactate + 1 equiv of NH_4^+ + H_2 equiv oxidized); H_2 equiv oxidized were calculated as the difference between H_2 supplied and H_2 recovered from headspace. [f] H_2 equiv incorporated calculated as (2 equiv of propionate + 2 equiv of butyrate + 4 equiv of valerate + 4 equiv of CH_4 + 3 equiv of nitroethane metabolized). The consumption of 3 H_2 equiv for complete reduction of nitroethane is based on the reported consumption of 3 H_2 for the reduction of 2-nitroethanol to 2-aminoethanol (*34*).

significant as are Pearson correlation coefficients for relevant variables. All data were analyzed using STATISTIX8 Analytical Software (Tallahassee, FL).

RESULTS

Methane, Hydrogen, and Carbon Dioxide. The main effects of treatment were observed on CH_4 production ($P < 0.05$), with all treated incubations producing less CH_4 (μmol mL^{-1} of incubation fluid) than control incubations (**Table 1**). Treatments containing 2× nitroethane (with or without monensin) showed a stronger antimethanogenic effect in comparison with monensin alone (**Table 1**), and this finding was evident upon comparison of least-squares means within incubation series (**Figure 1A**). An effect of treatment was also observed on CO_2 accumulation ($P < 0.05$), with lower concentrations produced in incubations treated with monensin, 2× nitroethane, or both inhibitors than in the controls (**Table 1**). All treatments decreased CO_2 accumulation after the first incubation series, but thereafter CO_2 was decreased more often in monensin-treated incubations, with or without added nitroethane, than in 2× nitroethane-treated incubations (**Figure 1B**). Quadradic effects ($P < 0.05$) on CO_2 accumulation during consecutive batch culture were observed for control and all treated incubations except those incubated with 2× nitroethane; linear effects were not observed ($P > 0.05$) for any of the incubations. A positive correlation ($P < 0.05$; Pearson correlation coefficient = 0.45) was observed between overall accumulation of CH_4 and CO_2. Within treatments, CH_4 and CO_2 accumulations were positively correlated (Pearson correlation coefficient) for controls (0.62; $P < 0.05$), 1× nitroethane (0.64; $P < 0.05$), 2× nitroethane (0.74; $P < 0.05$), and 2× nitroethane plus monensin (0.65; $P < 0.05$) but not monensin (0.15; $P > 0.05$) treatments due mainly to quadratic effects ($P < 0.05$), with decreased CO_2 accumulation observed early during the consecutive batch culture series (**Figure 1A**,**B**). A main effect of treatment was not observed on residual H_2 concentrations ($P > 0.05$) (**Table 1**), although less H_2 had been oxidized by monensin, 2× nitroethane, and 2× nitroethane plus monensin-containing treatments after the 6th and 16th series, respectively (**Figure 1C**).

Lactate and Ammonia. Small amounts of lactate and ammonia were produced during the incubations (**Table 1**), and in the case of lactate, a main effect ($P < 0.05$) of treatment was observed, with 2× nitroethane-treated incubations producing less lactate than controls but not in comparison to concentrations produced by 1× nitroethane, monensin, or 2× nitroethane plus monensin-treated incubations. Ammonia production (μmol mL^{-1}) was not affected by treatment ($P > 0.05$) (**Table 1**).

Volatile Fatty Acids and Fermentation Balance. Main effects of treatment were observed on butyrate, valerate, and isobutyrate but not on propionate or isovalerate production during consecutive batch culture of the mixed microbial populations (**Table 1**). Comparison of least-squares means revealed that considerable fluctuations in volatile fatty acid production occurred, particularly within incubations treated with 1× or 2× nitroethane (**Figures 2** and **3**). Concentrations of acetate and butyrate (**Figure 2A**,**C**) as well as isobutyrate and valerate (**Figure 3A**,**B**) produced after the third incubation series were considerably higher ($P < 0.05$) than those produced in controls or either monensin-treated incubation. Concentrations of propionate and isovalerate produced were also markedly increased within these nitroethane-treated incubations but not significantly so (**Figures 2B** and **3C**). Volatile fatty acid production by 1× and 2× nitroethane-treated incubations, but not necessarily by monensin and 2× nitroethane plus nitroethane-treated incubations, compared more closely with that by controls after the 6th and 10th incubations, particularly in the case of butyrate, isobutyrate, and valerate (**Figures 2** and **3**). The acetate/propionate ratio was affected ($P < 0.05$) by the 2× nitroethane plus monensin treatment in comparison to the controls (**Table 1**). Linear effects were observed ($P < 0.05$) on acetate to propionate ratios, and the production of butyrate by controls as well as on the production of isobutyrate by 1×

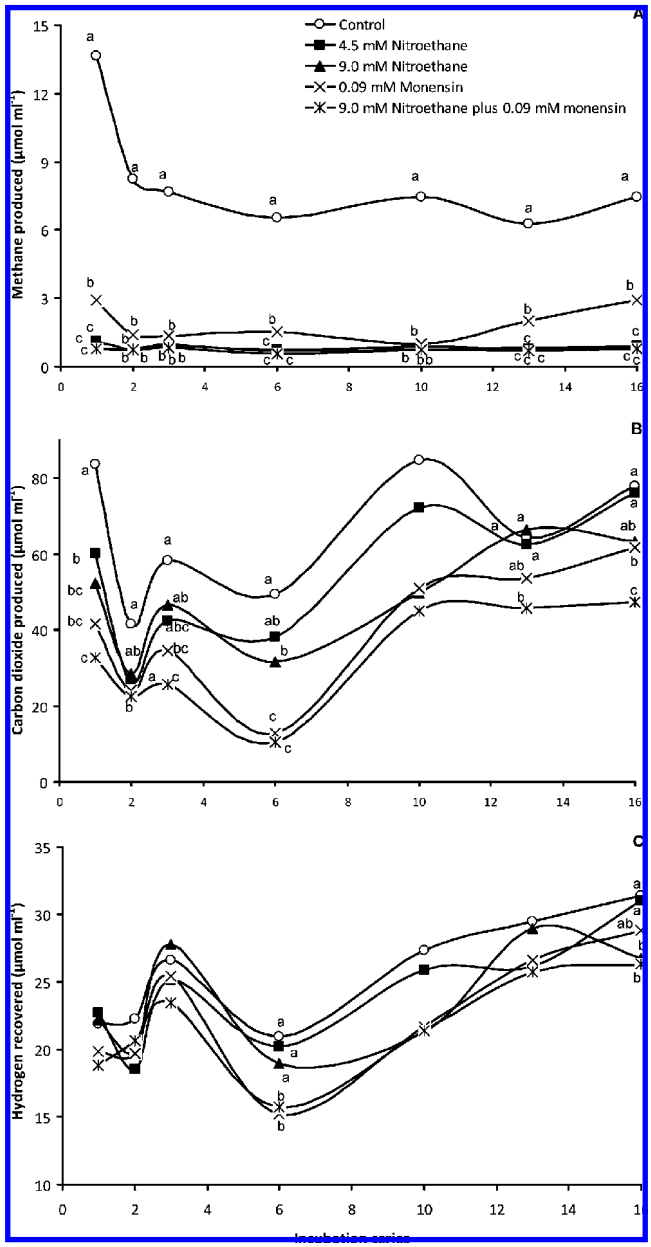

Figure 1. Effect of nitroethane and monensin on CH_4 and CO_2 accumulation and on residual H_2 concentration during consecutive batch culture of mixed populations of ruminal microbes. Values within series represent least-squares means, and those with unlike letters differ ($P < 0.05$). The SEM were 1.4, 5.3, and 1.1 for CH_4, CO_2, and H_2, respectively.

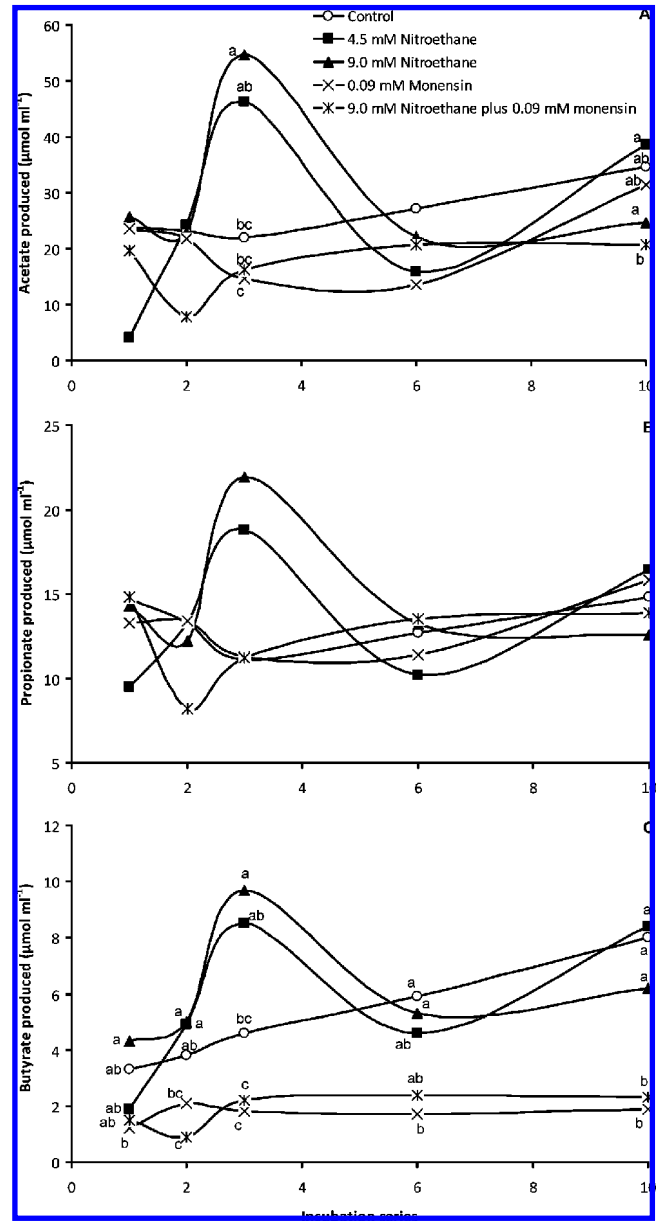

Figure 2. Effect of nitroethane and monensin on acetate, propionate, and butyrate production during consecutive batch culture of mixed populations of ruminal microbes. Values within series represent least-squares means, and those with unlike letters differ ($P < 0.05$). The SEM were 5.1, 1.6, and 1.0 for acetate, propionate, and butyrate, respectively.

nitroethane treatments during consecutive batch culture of monensin. A quadratic effect ($P < 0.05$) was observed on the production of butyrate during consecutive batch culture of monensin treatments.

Fermentation balance estimates of CH_4 production were not correlated with measured CH_4 accumulation ($P > 0.05$; Pearson correlation coefficient = 0.03). Estimates of CO_2 accumulation were correlated with measured CO_2 accumulation ($P < 0.05$; Pearson correlation coefficient = 0.28). Hydrogen balance estimates showed considerable disparity between amounts of reducing equivelants (expressed as μmol of H_2 equiv mL^{-1} of incubation fluid) (**Table 1**). Within incubations series, amounts of reducing equivalants generated during fermentation and oxidation nearly always exceeded amounts incorporated in fermentation products and metabolized nitroethane (**Table 2**). The discrepancy between reducing equivalants generated and incorporated was less ($P < 0.05$) for incubations treated with 2× nitroethane plus monensin than those treated with 1× nitroethane after the 2nd, 3rd, and 10th incubation series and less than those treated with monensin or 2× nitroethane after the 2nd and 3rd incubation series, respectively (**Table 2**). The discrepancy between reducing equivalants generated and incorporated was also less ($P < 0.05$) in control incubations after the third incubation series than for monensin-treated and 1× and 2× nitroethane-treated incubations. Linear or quadratic effects of treatments across incubation series were not observed ($P > 0.05$).

Nitroethane Degradation and Nitrometabolizing Bacterial Populations. Effects of nitroethane treatment on amounts of nitroethane metabolized are shown in **Table 1**, with more nitroethane being metabolized in incubations supplemented with

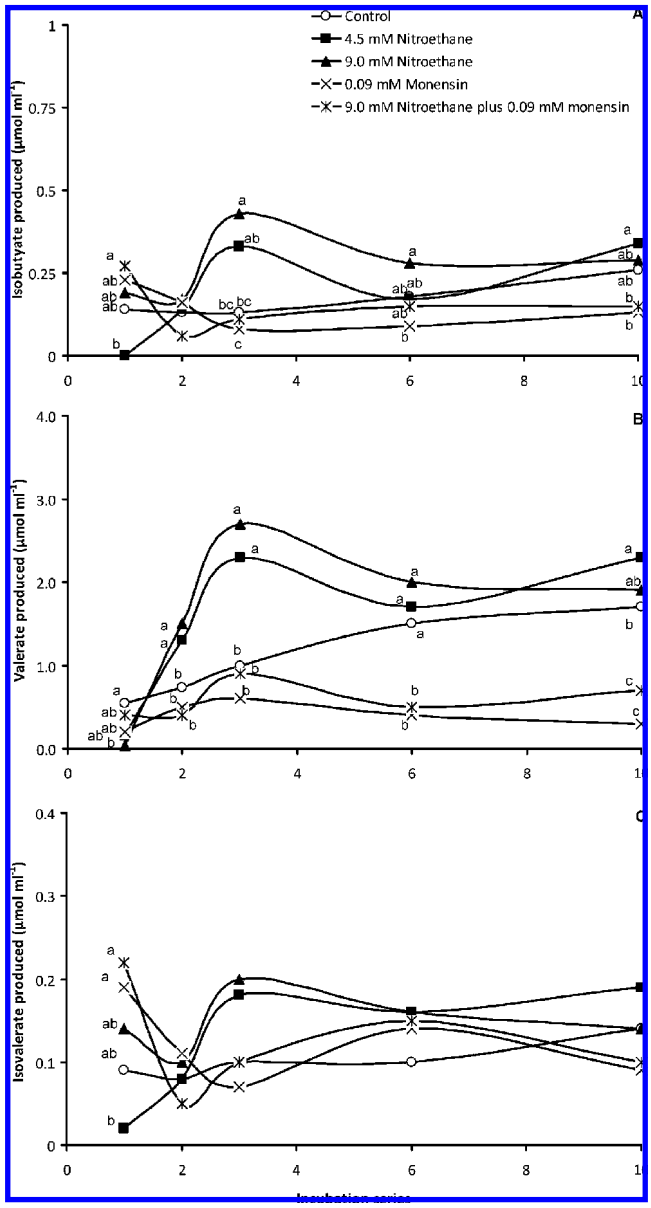

Figure 3. Effect of nitroethane and monensin on isobutyrate, valerate, and isovalerate production during consecutive batch culture of mixed populations of ruminal microbes. Values within series represent least-squares means, and those with unlike letters differ ($P < 0.05$). The SEM were 0.1, 0.3, and 0.1 for isobutyrate, valerate, and isovalerate, respectively.

higher concentrations (1× versus 2× and 2× plus monensin). More nitroethane ($P < 0.05$) was metabolized within 2× nitroethane than in 2× nitroethane plus monensin-treated incubations, indicating that nitroethane metabolism was negatively affected by monensin.

Most probable numbers of nitroreducing bacteria were increased ($P < 0.05$) in incubations treated with 1× and 2× nitroethane (6.9 and 5.9 $\log_{10}$ organisms mL^{-1}, respectively) at the sixth incubation series compared to control, monensin, and 2× nitroethane plus monensin treated incubations (2.5, 0.5, and 0.5 $\log_{10}$ organisms mL^{-1}). The SEM was 0.8.

Methane Production upon Removal of Inhibitory Pressure. When the mixed microbial populations were transferred into fresh medium lacking the inhibitory pressure exerted the previous 16 incubation series, CH_4 production by incubations treated with 1× nitroethane, 2× nitroethane, and 2× nitroethane plus monensin, but not by those treated with monensin alone, was lower ($P < 0.05$) than that produced by controls (**Figures 3** and **4**).

DISCUSSION

Results from the present consecutive batch culture experiment confirm the CH_4-inhibiting activity of nitroethane, although inhibition of CH_4 production was greater in nitroethane-supplemented incubations than in incubations containing monensin alone (by as much as 90 versus 78%, respectively) when compared to the control incubations. Moreover, results demonstrate that in the absence of added monensin the nitroethane treatments supported >1000-fold enrichment in most probable numbers of nitroreducing bacterial populations. The present incubations were conducted with H_2 added in excess so as not to limit access of the H_2-oxidizing populations to this H_2 substrate, and thus conditions for CH_4 production and nitroethane reduction were likely more favorable than what may be encountered within the rumen. However, earlier results indicated that enrichment of nitrometabolizing populations occurs within the rumen of nitroethane-treated animals as evidenced by increased rates of nitrocompound metabolism (*23*).

The increased rates of nitrocompound metabolism likely occur due to the enrichment of *D. detoxificans*, which at present is the only ruminal bacterium known to possess appreciable ability to metabolize the oxidized nitrocompounds (*29*). Whereas normally present at low numbers (10^4 cells mL^{-1}) within the rumen of animals having no exposure to the nitrocompounds, numbers of *D. detoxificans* can be enriched when provided a suitable electron acceptor (*36*). This bacterium is Gram-positive, which would explain its monensin sensitivity, and is an obligate nonfermentative anaerobe that conserves energy exclusively via anaerobic respiration, oxidizing H_2 and formate, as well as lactate, for the reduction of appropriate anaerobic electron acceptors. Suitable acceptors include oxidized short-chain nitrogen compounds such as nitroethane, 3-nitro-1-propionic acid, 3-nitro-1-propanol, 2-nitro-1-propanol, and 2-nitroethanol as well as nitrate, trimethyl amine-oxide, and dimethyl sulfoxide (*29*). Nitrate is reduced to ammonia by some strains of *D. detoxificans*, and the acceptors 3-nitro-1-propionic acid, 3-nitro-1-propanol are reduced to their respective amines, β-alanine, or 3-amino-1-propanol (*37*). Because reduction of these nitrocompounds by *D. detoxificans* consumes the major reductants used by ruminal methanogens, it is reasonable to suspect that apart from their chemical inhibition of ruminal methanogenesis, the nitrocompounds also have the potential to serve as alternative electron acceptors. In support of this hypothesis, addition of *D. detoxificans* strain NPOH1 and 20 mM nitrate to ruminal populations not previously exposed to nitrate caused a 90% decrease in CH_4 production compared to a decrease of <25% with nitrate alone, the latter due to induction and selection of endogenous nitrate-reducing populations during the 24 h incubation (*14*). The decrease in the amount of CH_4 produced was stoichiometrically equivalent to the amount of nitrate reduced to ammonia, with the reduction of CO_2 to CH_4 and nitrate to ammonia each consuming eight electrons (*14*).

In the present experiment, considerable amounts of nitroethane were metabolized ($3.9-8.23 \pm 0.1$ μmol mL^{-1}) within all nitroethane-treated incubations, consuming between 12 and 25 μmol of H_2 mL^{-1} of incubation fluid, which could account for approximately 3−6 μmol of CH_4 mL^{-1}. Because reductant was never limiting in the present incubations, as evidenced by the recovery of >15 μmol of H_2 mL^{-1} incubation fluid at the end of the incubations, we conclude that the decrease in CH_4

Table 2. Effects of Nitroethane, Monensin, or Their Combined Treatment on Hydrogen Balance Parameters during Consecutive Batch Culture of Mixed Populations of Ruminal Microbes

	μmol of H_2 equiv mL^{-1} of incubation fluid after treatment with[a]						
parameter[b]	control	1× NE	2× NE	Mon	2× NE + Mon	*P* value	SEM[c]
			First Series				
H_2 equiv generated[d]	95.2	42.4	101.4	86.0	82.4	0.15	15.8
H_2 equiv incorporated[e]	94.6 f	42.0 g	68.1 fg	43.7 g	63.1 g	0.0005	5.9
excess H_2 equiv (difference)	0.6	0.4	33.3	42.3	19.3	0.05	10.3
			Second Series				
H_2 equiv generated	87.2	105.1	100.9	85.3	46.9	16.10	0.1
H_2 equiv incorporated	72.2 f	58.3 fg	69.3 f	39.6 g	47.4 g	0.0028	5.3
excess H_2 equiv (difference)	15.0 fg	46.8 f	31.6 fg	45.7 f	−0.5 g	0.0271	11.5
			Third Series				
H_2 equiv generated	88.9 gh	165.6 fg	189.5 f	63.2 h	71.0 h	0.0035	20.3
H_2 equiv incorporated	67.9 fgh	81.2 fg	104.0 f	35.6 h	58.0 gh	0.0014	8.0
excess H_2 equiv (difference)	21.0 g	84.4 f	85.5 f	27.6 fg	13.0 g	0.0037	12.6
			Sixth Series				
H_2 equiv generated	113.2	84.2	104.8	70.1	89.6	0.44	19.2
H_2 equiv incorporated	71.1 d	52.7 fg	72.4 f	35.5 g	60.5 fg	0.0061	6.6
excess H_2 equiv (difference)	42.1	31.5	32.4	34.6	29.1	0.95	12.9
			Tenth Series				
H_2 equiv generated	133.3 fg	147.3 f	110.3 fg	104.3 fg	84.8 g	0.0439	15.1
H_2 equiv incorporated	84.3 f	76.3 fg	73.1 fg	42.2 h	63.6 g	0.0001	3.9
excess H_2 equiv (difference)	49.0 fg	71.0 f	37.2 fg	62.0 fg	21.2 g	0.0276	11.4

[a] Treatments: control; 1× NE, 4.5 mM nitroethane; 2× NE, 9.0 mM nitroethane; Mon, 0.09 mM monensin; and 2× NE plus Mon, 9.0 mM nitroethane plus 0.09 mM monensin. Values within rows with unlike letters differ ($P < 0.05$). [b] Parameters represent least-squares means from measuresments taken after series 1, 2, 3, 6, and 10. [c] SEM, standard error of the mean. [d] H_2 equiv generated calculated as (2 equiv of acetate + 1 equiv of propionate + 4 equiv of butyrate + 3 equiv of valerate + 1 equiv of lactate + 1 equiv of NH_4^+ + H_2 equiv oxidized); H_2 equiv oxidized were calculated as the difference between H_2 supplied and H_2 recovered from headspace. [e] H_2 equiv incorporated calculated as (2 equiv of propionate + 2 equiv of butyrate + 4 equiv of valerate + 1 equiv of lactate + 4 equiv of CH_4 + 3 equiv of nitroethane metabolized). The consumption of 3 H_2 equiv for complete reduction of nitroethane is based on the reported consumption of 3 H_2 for the reduction of 2-nitroethanol to 2-aminoethanol (*34*).

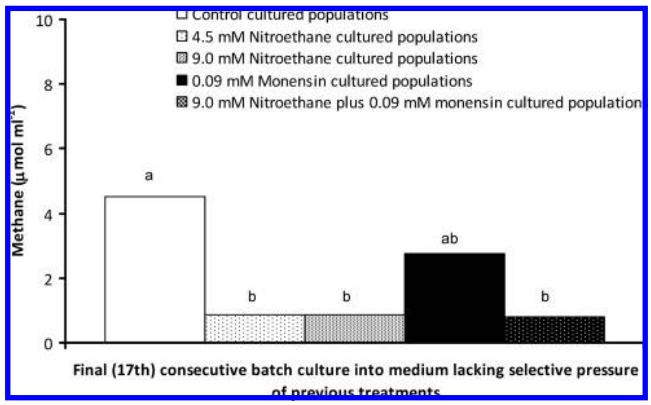

Figure 4. Effect of inhibitor removal on CH_4 production by mixed populations of ruminal microbes previously cultured over 16 consecutive 24 h incubations with respective treatments. Values represent least-squares means, and those with unlike letters differ ($P < 0.05$). The SEM was 1.3.

production was mainly due to a chemical effect rather than to consumption of reducing equivelants. Nitroethane, like several other short-chain nitrocompounds, has been shown to inhibit oxidation of formate by mixed populations of ruminal microbes under conditions of excess or limiting H_2 (*24*), but under the H_2 rich conditions of these consecutive batch cultures, H_2, rather than formate, would be expected to be the major substrate for methanogenesis. Formate accounts for approximately 18% of CH_4 produced in the rumen (*38*). It is possible that nitroethane may inhibit hydrogenase activity expressed by methanogens as a related nitrocompound, 2-nitroethanol, inhibited ferredoxin-linked hydrogenase activity of *Clostridium kluyveri* via its oxidation of ferredoxin (*34*). Conclusive evidence, however, that nitroethane or other nitrocompounds specifically inhibit hydrogenase activity of methanogens has not yet been demonstrated. Little is known regarding the biochemistry of formate and H_2 oxidation by *D. detoxificans*, but the fact that this bacterium uses these reductants as substrates to reduce the nitrocompounds suggests that the formate and H_2-oxidizing enzymes function differently from those expressed by the mixed population of ruminal microbes.

Consistent with earlier papers (*21–23*), nitroethane treatments in the absence of monensin did not markedly change proportions of the more abundant volatile fatty acids produced compared to that of controls. Similarly, monensin treatment, which typically results in increased propionate accumulation at the expense of CH_4 and acetate production (*39, 40*), did not affect amounts of acetate or propionate produced or the acetate/propionate ratio when administered without nitroethane. At H_2 concentrations >1 kPa, the ability of ruminal populations of microbes to dispose of excess reducing equivalants by hydrogenase catalyzed H_2 production can be inhibited (*2, 12*). Thus, at high H_2 concentrations, ruminal microbial populations compensate by redirecting the flow of fermentation-derived reducing equivalants to increased production of more reduced fermentation acids (*2*). Under the incubation conditions of this experiment, however, 1 kPa would be equivalent to 0.62 μmol of H_2 mL^{-1} incubation fluid, and therefore our provision of excess reductant likely caused increased production of more reduced acids such as propionate, which is maximally promoted at 2.4 kPa (equivalent to 1.48 μmol of H_2 mL^{-1} of incubation fluid) (*41*), even within control incubations, thus masking our ability to detect potential effects of nitroethane or monensin on propionate production. However, results from earlier studies indicate that in the case of nitroethane, alternative mechanisms other than the disposal of electrons via propionate production may be operative when low partial pressures of H_2 more typical

of the rumen environment are encountered. For instance, propionate production was not increased and small amounts of H_2 (<1.9 μmol mL^{-1} of rumen fluid) accumulated during in vitro incubations of ruminal fluid with nitroethane when 100% CO_2 was used as the gas phase (*21*). Additionally, although ruminal propionate concentrations were increased after several days of oral nitroethane or 2-nitroethanol administration to sheep and cattle, concentrations of acetate and butyrate were also increased proportionally, which further indicates that reducing equivalants were not redirected toward the production of propionate and butyrate with these treatments (*22, 23*).

Butyrate production was increased by 1× and 2× nitroethane treatments compared to that by controls, which conflicts with results from an earlier studies, where H_2 was not in excess, showing that nitroethane had no effect on butyrate accumulations in vitro (*21*) or in vivo (*22, 23*). However, butyrate production was also increased when mixed populations of ruminal microbes were incubated in vitro with excess reductant and additions of *D. detoxificans* plus nitrate (*14*). These findings suggest that ruminal populations enriched with *D. detoxificans* may potentially dispose of excess reducing equivalants via production of butyrate, which presumably would occur via interspecies interactions as *D. detoxificans* is nonfermentative and thus, other than producing acetate via the oxidation of lactate, has not been shown to produce fermentation acids (*42*). We cannot exclude, however, the possibility that butyrate may be formed within the incubations by the condensation of 2 units of acetate. Valerate production was increased >50% in 1× and 2× nitroethane treatments in comparison to controls, and this may represent another, albeit slight, disposal site for excess reducing equivalants.

Butyrate and valerate production were decreased by monensin and 2× nitroethane plus monensin treatments compared to that by controls, which is consistent with the reported effect of monensin against butyrate-producing and amino-acid fermenting bacterial populations (*39, 43, 44*). Monensin contained within the 2× nitroethane plus monensin treatment adversely affected populations of nitroreducing bacteria as their numbers were markedly decreased compared to the 1× and 2× nitroethane treatments. Our observed metabolism of nitroethane within all nitroethane-treated incubations, including those treated with 2× nitroethane plus monensin, suggests that the inhibitory effect of the ionophore was not complete in the consecutive batch culture medium. However, the inhibitory effect was apparent upon transfer of compromised or injured cells to the more restrictive most probable number enumeration medium.

Within the rumen, where H_2 concentrations are typically 1 μM (0.1 kPa) or less, formate as a fermentation product rarely accumulates but rather is rapidly catabolized to H_2 (*45*). Under conditions of excess reducing equivalants, however, some ruminal microbes can shift fermentation toward production of formate (*46*) and some can reduce CO_2 to formate (*47, 48*). It is possible that some of the excess reducing equivalants generated during our incubations may have been incorporated into formate and because nitroethane inhibits oxidation of formate, this product would be expected to accumulate to greater concentrations with nitroethane treatment. Formate accumulation has been observed within nitroethane-treated incubations of ruminal microbes with limiting amounts of added reductant and fermentable substrate (*24*). Lactate and ethanol are also potential products that may store reducing equivalants when H_2 concentrations are high, and our results show that lactate did accumulate at low, albeit measurable, concentrations. Lactate can be used as a substrate by the nitroreducing bacterium *D. detoxificans*, although less effectively than H_2 or formate (*42*), which may explain the slightly lower accumulation of lactate in incubations treated with 2× nitroethane. Ethanol was not measured in samples collected from our incubations, but it is reasonable to suspect that its accumulation may have been similar to that observed with lactate. As expected, ammonia production remained low within the consecutive batch culture incubations, and this was not affected by treatment. Increased H_2 concentrations can depress the production of ammonia (*49*). However, enhanced bacterial assimilation of branched-chain fatty acids or decreased proteolysis and amino acid deamination cannot be ruled out.

Measured amounts of CH_4 and CO_2 accumulation were positively correlated, but this was due mainly to quadratic effects associated with decreased CO_2 accumulation observed early during the consecutive batch culture series. After the 13th incubation series, CH_4 production began to increase in incubations treated with monensin alone, suggesting an adaptation of the monensin-treated populations to this inhibitor. The adaptation did not occur in incubations treated with 2× nitrotroethane plus monensin. Earlier studies have shown the CH_4-inhibiting activity of monensin begins to be decreased after approximately 14 days of monensin administration to cattle (*50*). Not unexpectedly, theoretical estimates of CH_4 production were not correlated with actual CH_4 production measurements because the stoichiometric equations do not account for the consumption of electrons consumed by nitroethane metabolism or incorporated into branched-chain fatty acids or lactate or the fate of up to 86 μmol of H_2 equiv generated during fermentation but not accounted for in our H_2 balance. Potentially, these H_2 equivalants could be incorporated into products such as ethanol, microbial protein, or possibly via assimilation of CO_2 into formate or acetate; however, the latter is less likely because estimated CO_2 production was correlated, albeit marginally, with measured CO_2 accumulation. It is also possible that some of the excess reducing equivalents may have accumulated intracellularly as reduced nucleotides.

The consecutive batch culture technique employed in this study provided an effective way to demonstrate that repeated exposure to nitroethane treatments resulted in an enrichment of nitroreducing bacterial populations. Limitations of this technique exist, however, as evidenced by the variable fermentation responses of the populations to the sporadic environmental changes experienced upon each successive incubation, particularly during the earlier incubation series. The marked decrease in CH_4 production after the first incubation series in untreated controls likely reflects a substantial decrease in protozoal numbers and diversity as these organisms, which are important symbiotic contributors to methanogenesis, which may not survive under our batch culture conditions (*51*). Moreover, the large fluctuations in volatile fatty acid production, particularly in the 1× and 2× nitroethane-treated cultures after the third incubation series likely reflect population fluxes in response to nonstatic nitroethane concentrations, which change at differing rates depending on numbers of nitrometabolizing bacteria and their phase of growth. The peak in VFA production was specific to the 1× and 2× nitroethane-treated cultures and was absent in the 2× nitroethane plus monensin-treated, which suggests that the response was attributable to the nitrometabolizing population that was monensin sensitive. The accumulation of CO_2 was markedly decreased early during the incubation series, which suggests fermentation of organic matter within the added alfalfa was decreased, likely due to high H_2 concentration in the headspace gas. However, we cannot exclude the possibility

that substantial amounts of CO_2 were fixed into products such as formate or acetate under the conditions of high H_2 concentrations. Nevertheless, our results confirm the CH_4-inhibiting activity of nitroethane and suggest that ruminal adaptation to low (<80 mg of nitroethane/kg of body weight per day), but not high level (160 mg of nitroethane/kg of body weight per day) treatment observed in earlier studies (*22, 23*) is likely due to an enrichment of nitrometabolizing bacteria. The 80 mg of nitroethane/kg of body weight per day dose was estimated to yield an intraruminal dose of approximately 7.2 μmol pf nitroethane mL^{-1}, and with rates of nitroethane metabolism increased from 0.05 to 0.15 μmol of nitroethane mL^{-1}, approximately half of the daily dose would have been metabolized after 24 h (*23*). Ruminal clearance rates of nitroethane in vivo, however, are likely much faster due to possible absorption, eructation, or fluid passage rate, contributing factors not encountered in our consecutive batch cultures, where average residual nitroethane concentrations ranged from 0.3 to 0.4, from 0.7 to 1.1, and from 0.9 to 1.7 μmol mL^{-1} for 1× and 2× nitroethane and 2× nitroethane plus monesin treatments, respectively. Thus, these processes combined with enrichment in ruminal metabolism likely contribute to depletion of ruminal nitroethane to concentrations no longer inhibitory to methanogenesis. Results from our final experiment, where the inhibitory pressure of nitroethane was removed by transferring previously treated populations to fresh medium lacking nitroethane, showed that CH_4 production by the populations previously treated with nitroethane remained very low compared to controls or to those previously treated with monensin. This finding suggests that methanogens were absent or had been diluted to very low numbers after 16 consecutive batch cultures of the mixed populations with nitroethane treatment, as it is unlikely that the CH_4-inhibiting activity of <0.2 μmol of nitroethane mL^{-1} carried over with the 10% inoculum could inhibit CH_4 production to that extent (*22*). Conversely, when transferred into the fresh medium lacking monensin, CH_4 production by populations that had previously been cultured over 16 consecutive incubations with monensin did not differ from that produced by controls having had no prior exposure to the inhibitory treatments. This finding suggests that methanogens were present or had recovered to sufficient numbers within the monensin-treated populations, lacking nitroethane, to produce appreciable amounts of CH_4 despite the inhibitory pressure of monensin encountered in the 16 previous incubation series.

From a practical standpoint, nitroethane is likely not an attractive candidate for use as a commercial feed supplement to reduce ruminal CH_4 production, primarily because of concerns regarding the toxicity of its presumed reduction product, ethanamine. Although toxic effects were not observed in rats following 2 years of chronic inhalation exposure to 100 or 200 ppm of nitroethane (*52*), adverse effects on liver, kidney, and lung tissues were observed in rabbits following a 6 week chronic exposure to 100 ppm of ethanamine (*53*). Existing evidence indicates that, unlike secondary nitroalkanes such as 1-nitrobutane and 1-nitropropane, the primary nitroalkanes such as nitroethane and nitrocarbinols are not carcinogenic on the basis of the Ames *Salmonella* assay (*54*). However, more research is needed to fully assess its potential genotoxicity. Also of importance from a practical standpoint is that the reductant conserved in ethanamine may have limited value for the ruminant host. The rationale for using nitroethane in our studies was that it was the most effective of commercially available compounds tested (*22, 23*). More recently, our research efforts have focused on testing other nitrocompounds that are also efficacious in decreasing ruminal CH_4 production but that upon reduction will yield nontoxic amino acid products with potential nutritional value for ruminant animal. Such work may ultimately yield practical strategies to minimize the negative contribution of ruminal methanogenesis to ruminal digestion and atmospheric CH_4 emissions.

ACKNOWLEDGMENT

We thank Jacquiline Kotzur for expert technical assistance.

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Received for review March 11, 2008. Accepted March 17, 2008. We thank the Mexican government program "PROMEP" for the scholarship provided for H.G.-B.

JF800756C