Effects of select nitrocompounds on in vitro ruminal fermentation during conditions of limiting or excess added reductant

Robin C. Andersona,*b, Nathan A. Kruegera, Thaddeus B. Stantona, Todd R. Callawaya, Thomas S. Edringtona, Roger B. Harveya, Yong Soo Junga, David J. Nisbetta, Robin C. Andersona

aUnited States Department of Agriculture, Agricultural Research Service, Food and Feed Safety Research Unit, 2881 F&B Road, College Station, TX 77845, USA
bPre-Harvest Food Safety and Enteric Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, 2300 North Dayton Avenue, Ames, IA 50010, USA

Article history:
Received 16 January 2008
Received in revised form 31 March 2008
Accepted 5 April 2008
Available online 5 June 2008

Abstract

Ruminal methane (CH4) production results in the loss of up to 12% of gross energy intake and contributes nearly 20% of the United States’ annual emission of this greenhouse gas. We report the effects of select nitrocompounds on ruminal fermentation after 22 h in vitro incubation (39 °C) with or without additions of hydrogen (H2), formate or both. In incubations containing no added reductant, CH4 production was inhibited 41% by 2-nitro-1-propanol (2NPOH) and >97% by 3-nitro-1-propionic acid (3NPA), nitroethane (NE) and 2-nitroethanol (2NEOH) compared to non-treated controls and H2 did not accumulate. With formate as the sole added reductant, nitro-treatment reduced CH4 production by >95% and caused 42% to complete inhibition of formate catabolism compared to controls, and the accumulation of H2 increased slightly. Nitro-treatment decreased CH4 production 57–98% from that of controls when supplied H2 or formate plus H2. Formate catabolism was decreased 42–84% from that in controls by all nitro-treatments except 3NPA with both formate and H2. Greater than 97% of the added H2 was catabolized within controls; >84% was catabolized in nitro-treated incubations. Acetate, propionate and butyrate accumulations were unaffected by nitro-treatment irregardless of reductant; however, effects on ammonia and branched chain fatty acid accumulations varied. These results suggest that nitro-treatment inhibited formate dehydrogenase/formate hydrogen lyase and hydrogenase activity.

Published by Elsevier Ltd.

1. Introduction

Ruminal methanogenesis results in the loss of up to 12% of gross energy intake for forage fed cattle and up to 4% for concentrate fed cattle (Johnson and Johnson, 1995). Environmentally, ruminal methanogenesis contributes nearly 20% of the United States’ total emissions of this greenhouse gas (EPA, 2006). Whereas, ruminant microbiologists have attempted to develop methods to reduce energetic losses associated with ruminal methane (CH4) production, only the use of ionophores such as monensin and lasalocid has been widely implemented, typically achieving transient reductions in CH4 production of approximately 30% (Russell and Strobel, 1989; Van Nevel and Demeyer, 1996). In many cases, reductions in ruminal CH4 production also adversely affected digestive function and microbial cell yields because the inhibition of CH4 production also diminished fermentation efficiencies associated with microbial interspecies hydrogen (H2) transfer reactions (Miller, 1995; Van Nevel and Demeyer, 1996). Recently, we have shown that 2 mM nitroethane (NE) inhibited CH4 production in vitro by more than 50% and that 12 mM NE or 2-nitro-1-propanol (2NPOH) each inhibited CH4 production in vitro by more than 90% (Anderson et al., 2003). Unlike some other CH4 inhibition strategies, however, NE nor 2NPOH had little effect on molar proportions of volatile fatty acids produced by ruminal populations and although acetate concentrations were sometimes reduced, total volatile acid production was increased (Anderson et al., 2003; Gutierrez-Bañuelos et al., 2007a). Thus, a major obstacle that could limit the use of the nitrocompounds as CH4 inhibitors appears not to exist since fermentation efficiencies associated with microbial interspecies H2 transfer were not compromised in these studies. Little is known regarding the mechanism by which the nitrocompounds inhibit methane production. This study was conducted to assess the effects of several different short chain nitrocompounds on catabolism of the main reductants, hydrogen (H2) and formate, used during ruminal methanogenesis as well as on accumulations of CH4, volatile fatty acids and ammonia.
2. Methods

2.1. In vitro incubations

Ruminal fluid was collected at 08:00 before appreciable morning grazing activity, so as to lessen the contribution of endogenous fermentable substrates to the pool of reducing equivalents during subsequent in vitro incubation, from a cannulated cow pastured on rye grass and having no known exposure to the nitrocompounds. The freshly collected ruminal fluid was strained through a sterilized nylon paint strainer (Leyendecker et al., 2004) into a 1 L receiving vessel. The vessel was filled with strained ruminal fluid, closed with a rubber stopper and returned to the laboratory for distribution (10 ml volumes) under a continuous stream of either 100% CO2 or 80% H2 in CO2 to 18 \( \times \) 150 mm crimp-top tubes preloaded with small volumes (<5% vol/vol) of distilled water or concentrated stock solutions (in water) of nitrocompounds or sodium formate. Separate experiments were conducted concurrently with supplements to achieve 0 or 12 \( \mu \)mol ml\(^{-1}\) each 2NPOH, NE, 3-nitro-1-propanic acid (3NPA) or 2-nitroethanol (2NEOH) under conditions of no added reductant, 60 \( \mu \)mol ml\(^{-1}\) added formate, 60 \( \mu \)mol ml\(^{-1}\) added H2 or additions of both H2 and formate (each at 60 \( \mu \)mol ml\(^{-1}\)). Upon addition of ruminal fluid, each tube was immediately fitted with a rubber stopper to contain the respective gas phase, crimped closed and incubated upright at 39 °C for 22 h without agitation. The pH of the strained ruminal fluid was measured with a pH meter at the time of distribution and was 6.30 and 6.80 after equilibration with 100% CO2 or H2:CO2 (80:20), respectively.

2.2. Analysis

Gas volume was determined in each tube at the end of the incubation period by measuring volume displacement in a 30 cc lubricated air-tight glass syringe fitted with an 18 gauge needle and then inserted through the stopper of each tube. Gas composition in samples collected from the headspace was determined by gas chromatography (Allison et al., 1992). Fluid samples were collected upon initiation and immediately after gas analysis for colorimetric determination of nitrocompounds (Anderson et al., 1993; Majak et al., 1986) and ammonia (Chaney and Marbach, 1962). The fluid samples collected after gas analyses were also analyzed for concentrations of volatile fatty acids by gas chromatography (Salanitro and Muirhead, 1975).

2.3. Statistics

All incubations were performed in triplicate. Data within each experiment were analyzed by general analysis of variance and Tukeys separation of means using Statistica®8 Analytical Software (Tallahassee, FL, USA).

3. Results and discussion

3.1. Effects on methane production

Consistent with earlier in vitro and in vivo studies (Anderson and Rasmussen, 1998; Anderson et al., 2003, 2006; Gutierrez-Baluetos et al., 2007a; Saengkerdsub et al., 2006), the short chain nitrocompounds 2NPOH, 3NPA, NE and 2NEOH markedly inhibited CH4 production. In the incubations containing no added reductant, 4.2 ± 0.2 \( \mu \)mol CH4 mol\(^{-1}\) incubation fluid was produced after 22 h incubation in the controls containing no added nitrocompound indicating that approximately 16.8 \( \mu \)mol ml\(^{-1}\) of endogenous reducing substrate was available to support methanogenesis.

Amounts of CH4 produced (\( \mu \)mol ml\(^{-1}\) incubation fluid) in incubations containing no added reductant were reduced to 42% from that of non-nitro supplemented controls in incubations supplemented with 2NPOH and reduced to near the lower limits of detection (0.10 \( \mu \)mol ml\(^{-1}\)) in those supplemented with the other nitrocompounds (Fig. 1A). These results support those from earlier studies indicating that NE and 2NEOH were nearly equally effective in inhibiting ruminal CH4 production in vitro (Anderson et al., 2003) and that NE inhibited CH4-producing activity more effectively than 2NPOH in the ovine rumen (Anderson et al., 2006). However, NE inhibited CH4 production to a much greater extent than 2NEOH during in vitro incubation of chicken cecal contents (Saengkerdsub et al., 2006), thus implicating potential differences in sensitivity to the nitrocompounds by methanogens endogenous to the different gut populations. Evidence suggests that the methanogen populations inhabiting ruminants are more diverse than that in chickens, the later which appear to be colonized predominately, if not exclusively, by Methanobrevibacter woesei (Jarvis et al., 2000; Nicholson et al., 2007; Saengkerdsub et al., 2007a,b; Whitford et al., 2001; Wright et al., 2004; Yangaita et al., 2000). When the reductant was supplied as formate alone, all nitrocompounds decreased CH4 production >98% from that produced by untreated controls (Fig. 1A). Conversely, nitro-supplementation decreased CH4 production 57–98% from that of controls containing no added nitrocompound when supplied H2 or formate plus H2, with CH4 inhibited to the greatest extent with NE and 2NEOH, intermediate with 3NPA and least with 2NPOH (Fig. 1A).

3.2. Effects on reductant oxidation and nitrocompound metabolism

Hydrogen concentrations differed slightly, or not at all, between non-nitro supplemented and nitro-supplemented incubations containing no added reductant or reductant added as formate alone, averaging 0.23 ± 0.1 and 1.07 ± 0.6 \( \mu \)mol H2 ml\(^{-1}\) of incubation fluid, respectively (Fig. 1B). In incubations supplemented with formate alone, essentially all the 60 \( \mu \)mol ml\(^{-1}\) added formate was catabolized in controls containing no added nitrocompound (Fig. 1C). This was as expected as formate is usually catabolized very rapidly and rarely accumulates to appreciable concentrations within unperturbed rumen incubations. Formate catabolism occurs largely by the activity of formate dehydrogenase/formate hydrogen lyase of methanogens and non-methanogens, the latter to CO2 and H2 (Asanuma et al., 1998; Hungate et al. 1970). Hydrogen liberated by this activity is rapidly oxidized by methanogens for the reduction of CO2 to CH4 (Asanuma et al., 1998; Hungate et al. 1970). Some ruminal methanogens also oxidize formate directly via the activity of a formate dehydrogenase, and it has been estimated that 18% of the ruminal CH4 produced is derived from formate (Hungate et al. 1970; Stewart et al., 1997). In the experiments reported here, >90% of the electrons derived from the amount of formate catabolized could be accounted for in the amount of methane produced in the non-nitro supplemented controls. Conversely, formate concentrations remained high, 103%, 64%, 42% and 118% of the 60 \( \mu \)mol ml\(^{-1}\) that was added, in incubations supplemented with 2NPOH, 3NPA, NE and 2NEOH, respectively, thus implicating an inhibition of formate dehydrogenase/formate hydrogen lyase activity as potential mechanisms of action of the nitrocompounds (Fig. 1C). This hypothesis is consistent with the nearly complete inhibition of methanogenesis observed in the nitro-supplemented incubations containing formate as the sole added reductant. The low accumulation of H2 in these nitro-supplemented incubations is also consistent with a suspected inhibition of H2 production by formate dehydrogenase/formate hydrogen lyase activity as in the absence of CH4 production, H2 would otherwise have been expected to accumulate to appreciably higher concentrations. A net gain of about 2–11 \( \mu \)mol formate ml\(^{-1}\) was measured from incuba-
tions supplemented with 2NPOH and 2NEOH, which suggests that under these conditions formate was produced as an endproduct of endogenous fermentation. Under the conditions of excess reducing equivalents, some ruminal microbes can reduce CO\textsubscript{2} or shift fermentation towards the production of formate (Asanuma et al., 1999; Miller and Wolin, 1973; Shi et al., 1997). Some of the formate, whether added or endogenous, may have been catabolized for reduction of the varying amounts of nitrocompounds metabolized (Fig. 2) although other reductants such as H\textsubscript{2} or lactate could support these reductions. The fate of the electrons liberated from the 34.8 μmol formate ml\textsuperscript{-1} catabolized in the NE-supplemented incubations is unknown as none of the added NE was reduced at the end of these incubations (Fig. 2). At present, the only known ruminal bacterium possessing appreciable nitroalkane-metabolizing activity is *Denitrobacterium detoxificans*, an obligate non-fermentative anaerobe that conserves energy via respiration. *D. detoxificans* oxidizes H\textsubscript{2}, formate or lactate for the reduction of nitrate, 3NPA and 3-nitro-1-propanol as well as trimethyl amine oxide, dimethyl sulfoxide and other short chain nitrocompounds, including those tested here, although reducing substrates have not been fully characterized for all of these acceptors (Anderson et al., 2000). While usually present at low numbers (approximately 10\textsuperscript{4} cells ml\textsuperscript{-1} ruminal fluid) in animals having no exposure to the nitrocompounds, the population can be enriched via supplementation with a suitable electron acceptor such as 3-nitro-1-propanol (Anderson et al., 1996). Numbers of nitro-reducing bacteria can be enriched under some conditions when exposed to NE as evidenced by increased rates of NE-reduction and increased numbers of nitro-reducing bacteria within mixed populations of ruminal bacteria (Gutierrez-Bañuelos et al., 2007a,b); however, the conditions during their enrichments contained no added formate. In this study, formate appears to have been inhibitory to the reduction of

![Fig. 1. Amounts (per ml of incubation fluid) of methane (A), hydrogen (B) and formate (C) after 22 h in vitro incubation of ruminal microbes in rumen fluid with or without added reductant (formate, hydrogen or both) and (or) nitrocompound. Reductants were supplied each to achieve 60 μmol ml\textsuperscript{-1}, and nitrocompounds were supplied each to achieve 12 μmol ml\textsuperscript{-1} of incubation fluid. Mean ± SD (n = 3) within the conditions of differing reductant with unlike letter designations differ (P < 0.05).](image-url)
NE even when H₂ was supplied as no NE was reduced in incubations containing both reducing substrates but approximately 1.1 μmol NE ml⁻¹ was reduced in incubations containing no added reductant or H₂ as sole added reductant (Fig. 2). Little is known regarding the biochemistry of nitroalkane reduction by *D. detoxificans*. Consequently, it is not known if formate or H₂ is utilized equally well as reductants for some of the tested nitrocompounds or whether some of the nitrocompounds may be reduced more easily or support enrichment of *D. detoxificans* more readily than the other nitrocompounds.

The presence of H₂ alleviated the inhibitory effect on formate oxidation as more formate was catabolized in the incubations supplemented with 2NPOH, 3NPA and 2NEOH when both H₂ and formate were supplied (each at 60 μmol ml⁻¹) as reductants than in incubations containing formate as the sole added reductant (Fig. 1C). In the case of incubations supplemented with 2NPOH, 3NPA and 2NEOH, at least some of the electrons derived from the oxidation of the additional formate (34–37 μmol ml⁻¹) in the incubations containing both added formate and H₂ could possibly have been used to support reduction of the 2.4, 6.7 and 0.07 μmol ml⁻¹ nitrocompound metabolized, respectively (Fig. 2). Similar to that observed in incubations containing formate as the sole added reductant, NE was not reduced in incubations with both H₂ and formate added. The fate of the remaining electrons derived from formate oxidation is uncertain as it is the fate of most of the electrons derived from the oxidation of >83% of the added H₂. For instance, the production of CH₄ in the incubations containing both added H₂ and formate would account for 33.7, 14.3, 8.2, 1.4 and 1.6 μmol reducing substrate ml⁻¹ by control, 2NPOH, 3NPA, NE and 2NEOH-supplemented incubations, respectively. Considering that CH₄ production from formate was essentially abolished in nitro-supplemented incubations containing formate as the sole added reductant, it is probable that electrons derived from the oxidation of H₂ contributed to the production of CH₄ when H₂ was present. Moreover, formate dehydrogenase activity by some methanogens is suppressed in the presence of H₂ (Sparling and Daniels, 1990; Wood et al., 2003). This conclusion is supported by the observation that amounts of H₂ catabolized and CH₄ produced in incubations containing both added formate and H₂ were strikingly similar to those produced in the respective nitro-supplemented incubations containing H₂ as the sole added reductant. The observation that less CH₄ accumulated in control incubations supplied both formate and H₂ (8.4 ± 1.3 μmol ml⁻¹ incubation fluid) than in incubations supplied with each reductant separately is not unexpected as formate at concentrations between 50 and 100 mM inhibited growth and CH₄ production of some, but not all, methanogens when under a H₂:CO₂ (50:50) gas phase (Beelay et al., 1986).

Hydrogen is usually present at approximately 1 μmol (0.1 kPa) in the unperurbed rumen (Hungate, 1975; Thauer et al., 1977); however, concentrations often increase to the concentrations that can inhibit hydrogenase activity (1 kPa) when ruminal CH₄ production is inhibited due to decreased H₂ consumption by methanogens (Miller, 1995; Van Nevel and Demeyer, 1996). Under the incubation conditions in this study, 1 kPa is equivalent to 0.62 μmol H₂ ml⁻¹ incubation fluid; thus it is probable that H₂ concentrations inhibited hydrogenase activity in all the nitro-supplemented incubations except those incubated without added reductant. Hydrogen accumulated to concentrations high enough to inhibit hydrogenase as well in the non-nitro supplemented controls incubated with added formate only. 2-Nitroethanol also inhibits ferredoxin-linked hydrogenase activity of *Clostridium kluyveri*, via the oxidation of ferredoxin (Angermairer and Simon, 1983), but it is not clear if 2NEOH or the other nitrocompounds similarly inhibited electron carrier-linked activity of the methanogens, which do not utilize ferredoxin as an electron carrier (Deppenmeier, 2002). In the present H₂-supplemented incubations (with or without added formate), H₂ concentrations were 5.5 to 9.2 μmol H₂ ml⁻¹ higher (*P < 0.05*) when nitrocompounds were present, but still considerable catabolism (>50 μmol H₂ ml⁻¹ incubation fluid) of the added H₂ occurred. Incubation of ruminal microbes under a 50%H₂:25%N₂ gas phase with the hydrogenase inhibitor carbon monoxide (CO; at 25%) also decreased CH₄ production by >85%, and in this case catabolism of the added H₂ was essentially complete as final concentrations were below detectable limits after 34 h incubation (Russell and Jeraci, 1984).

### 3.3. Effects on volatile fatty acid accumulation and ammonia production

As H₂ accumulates, the microbial ecosystem compensates by disposing reducing equivalents generated during fermentation towards increased production of more reduced fermentation end products, notably propionate (Van Nevel and Demeyer, 1996). Consequently, a possible disposal site for electrons generated during oxidation of formate and H₂ could be the production of increased concentrations of more reduced fatty acids. Analysis of volatile fatty acids at the end of the 22 h incubation in this study revealed no effect (*P > 0.05*) of nitro-treatment, irregardless of added reductant, on accumulations of the more abundant volatile fatty acids.

---

**Fig. 2.** Amounts (per ml of incubation fluid) of nitrocompound metabolized after 22 h in vitro incubation of ruminal microbes in rumen fluid with or without added reductant (formate, hydrogen or both) and (or) nitrocompound. Reductants were supplied each to achieve 60 μmol ml⁻¹, and nitrocompounds were supplied each to achieve 12 μmol ml⁻¹ of incubation fluid. Mean ± SD (*n* = 3) within the conditions of differing reductant with unlike letter designations differ (*P < 0.05*).
produced when compared to controls and this was reflected by no observable effect on the sum accumulation of total volatile fatty acids and only minor effects on the ratio of acetate to propionate (Table 1). In the case of acetate, mean (± SD) concentrations were...
4. Conclusion

Results from this study strongly implicate that the nitrocompounds are potent inhibitors of formate dehydrogenase/formate hydrogen lyase activity. This activity is likely inclusive of both methanogenic and non-methanogenic bacteria as evidenced by near complete inhibition of both CH₄ and H₂ production from added formate as the sole reductant. In the presence of H₂ added in excess, CH₄ production was also inhibited by these short chain nitrocompounds, with inhibition greatest with 2NEOH and NE and least with 2NPOH. In comparison to earlier work, effects of the nitrocompounds on CH₄ production, H₂ catabolism, volatile fatty acid and ammonia production were more similar to those observed with the hydrogenase inhibitor carbon monoxide (Russell and Jeraci, 1984) than those observed with the co-enzyme M inhibitor 9,10-anthraquinone and the F₄₂₀ inhibitor 2-bromoethanesulfonic acid. Unexpectedly, accumulations of isobutyrate, isovalerate, valerate and ammonia were higher in incubations containing added H₂, with or without added formate, than in incubations containing no added H₂ (Figs. 3 and 4). Increased H₂ concentrations sometimes, but not always, depress the production of branched chain volatile fatty acids and ammonia (Hino and Russell, 1985; Russell and Martin, 1984). It is possible that the higher initial pH in the incubations containing 80% H₂ (in CO₂) relative to those under a 100% CO₂ gas phase may have contributed to the higher amounts of ammonia produced as rates of ammonia production are more rapid at higher pH, particularly with forage fed animals (Lana et al., 1998). The amounts of ammonia produced in the present incubations were low, however, relative to amounts produced in an earlier study conducted with a 100% CO₂ gas phase and added formate. In the earlier study, 2NPOH, NE and 2NEOH treatment either increased or had no effect on ammonia accumulations (Anderson et al., 2003). The low amount of ammonia produced in the present incubations could possibly be due to low endogenous concentrations of degradable nitrogenous substrate. In all the nitro-supplemented incubations containing added formate, whether alone or with H₂, accumulations of isovalerate were markedly decreased when compared to controls. Likewise, NE and 2NEOH reduced the accumulations of isobutyrate in incubations containing both added formate and H₂ and 2NEOH reduced the accumulations of isobutyrate and isovalerate in incubations containing H₂ as the sole added reductant. Unfavorably high NADH/NAD ratios, as encountered during conditions of increased H₂ concentrations, inhibit fermentation and subsequent deamination of reduced but not neutral or oxidized amino acids (Hino and Russell, 1985). Thus, decreased accumulations of ammonia and the branched chain fatty acids, products of amino acid fermentation and deamination (Allison, 1978; Wallace et al., 1997), possibly reflect increased intracellular accumulations of NADH, which may account for some of the electrons derived from catabolism of the added reductants.
onic acid (Garcia-Lopez et al., 1996; Martin and Macy, 1985). These findings provide insight into the mechanistic activity of the nitrocompounds and ultimately may facilitate the development of commercially viable interventions to reduce economic and environmental costs associated with rumen methane production.

Acknowledgements

We thank Deb Lebo, Jackie Kotzur and Chelsea Pratt for their expert technical assistance.

References


