Metabolism of the plant toxins nitropropionic acid and nitropropanol by rumen microorganisms

by

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GENERAL INTRODUCTION

Astragalus species that synthesize the toxic aliphatic nitro compounds 3-nitro-1-propanol (NPOH) or 3-nitro-1-propionic acid (NPA) are responsible for poisoning thousands of cattle and sheep each year (49). Economic losses due to reduced weight gains and death can be significant (14, 35, 56). Other species within the legume family, such as some *Coronilla*, *Indigofera*, and *Lotus* species as well as certain species within the Malpighiaceae, Corynocarpaceae, and Violaceae families, also synthesize NPA (27). The nitro-toxins commonly exist as glucose conjugates. Ten esters of NPA have been identified (9, 16, 27), and the most common ether glycoside of NPOH, miserotoxin (3-nitro-1-propyl-β-D-glucopyranoside), was first isolated and identified by Stermitz et al. (46). Because ruminal microbes possess both esterase and β-glycosidase activity, the conjugates are rapidly hydrolyzed within the rumen to glucose and the respective nitro-toxin (27).

Ruminal microorganisms have been shown to metabolize free NPA or NPOH but the details concerning this metabolism remain unclear. The metabolism of NPA by ruminal microbes occurs at a much faster rate than that of NPOH (10, 22, 17) which may explain why ruminants are less susceptible to

poisoning by NPA than NPOH (22, 27). In this regard, it has been demonstrated that the rumen environment can be manipulated to enhance degradation of NPOH. Majak et al. (19) observed significant differences in the rate of microbial NPOH metabolism when rumen microbes were collected from cattle consuming different forages. They suggested that increased rates of NPOH metabolism may result from selection of competent microbes by the presence of unidentified NPOH analogs in some forages or because of the availability of unspecified hydrogen donors. Differences between forage appear not to be due simply to dietary energy content, however, because the rate of microbial NPOH metabolism was not significantly affected by energy supplementation (19). In another study, the rate of microbial NPOH metabolism was increased 4-fold when cattle diets were supplemented with nitroethane (20 mg/kg body wt), a nontoxic analog of NPOH. These results suggest either that NPOH metabolic activity is induced or that the microbial population itself adapts to the presence of the analog (20).

A correlation has been observed between microbial NPA and NPOH metabolism and microbial nitrite reduction (17). This suggested that NPA and NPOH degradation proceeds via a reductive cleavage of the nitrite group from each parent compound. Additionally, nitrite was detected in resting

cell cultures of rumen microorganisms incubated with NPA or NPOH. Evidence supporting the reductive cleavage mechanism was not conclusive, however, because the nitrite yield was less than 5% in resting cell cultures (17).

The present study was designed to define some of the microbial processes involved in the metabolism of NPA and NPOH. An understanding of the mechanistic processes and of the microbial populations involved in the metabolism of NPA and NPOH may lead to the development of practical methods to prevent livestock poisonings.

Explanation of Thesis Format

The alternate thesis format has been used for this thesis. A general introduction and literature review precede the manuscript, which will be submitted for publication to Applied and Environmental Microbiology, followed by a general summary and dicussion. The literature cited in the general introduction, literature review, and summary and discussion sections follows the summary and discussion. The thesis conforms to the style required by Applied and Environmental Microbiology.

LITERATURE REVIEW

Distribution

Several genera of the legume family are known to synthesize 3-nitro-1-propionic acid (NPA). These include *Indigofera*, *Coronilla*, *Lotus*, and *Astragalus* species (27). Additionally, certain members of the Malpighiaceae, Corynocarpaceae, and Violaceae families also synthesize NPA (27). Moreover, some *Astragalus* sp. synthesize 3-nitro-1-propanol (NPOH) (27) and these have received attention because of reported livestock poisonings (14, 56).

Worldwide, more than 450 species and varieties of *Astragalus* synthesize either NPA or NPOH (47, 49, 50). Of these, more than 50% (263 species and varieties) occur in North America alone (49, 50) with poisonings being reported primarily in the Rocky Mountain region (14, 48, 49, 54, 56). The NPOH-synthesizing species, *Astragalus miser* var. *oblongifolious* (Rydb.) Cron., *A. miser* var. *hylophilus* (Rydb.) Barneby, and *A. miser* var. *serotinus* (Gray) Barneby, are the most commonly blamed in livestock poisonings. In all, 20 species and 8 varieties in 10 taxonomic sections of North American *Astragalus* synthesize NPOH (48). Most of the South American species examined synthesize NPOH (48). Of the Old World *Astragalus*, 10 species in 5 sections

synthesize NPOH while 37 species in 19 sections and one subgenus synthesize NPA (48). Most of the Old World nitro-containing *Astragalus* occur in the U.S.S.R. or the Middle East (48). Only recently has an *Astragalus* been detected that synthesizes both compounds (51). Williams and Barneby (49, 50) demonstrated that chemotaxonomic relationships existed among species of *Astragalus* and that these relationships could be used to predict the occurrence and even the type of aliphatic nitro compound present in untested species.

The nitro alcohol is considered to be more poisonous than NPA to ruminants (9, 10). In sheep for example, the oral lethal dose of NPOH was 50 mg nitrite/kg body weight whereas the oral lethal dose of NPA was 125 mg nitrite/kg body weight (52). However, the quantity of aliphatic nitro compound may be higher in NPA-containing *Astragalus* than in NPOH-containing *Astragalus* (48). For instance, the NPOH-containing species *A. pterocarpus* Wats., *A. convallarius* Greene, and *A. diversifolius* Gray contained less than 8 mg nitrite/g plant whereas the NPA-containing species *A. cibarius* Sheld and *A. canadensis* var. *brevidens* (Gand.) Barneby contained 16 and 36 mg nitrite/g plant, respectively (52). Furthermore, the NPA-containing *A. falcatus* Lam. was determined to contain 84 mg nitrite/g plant (49). Consequently, it has been suggested that NPA-containing plants

may be as toxic per gram of plant material as NPOH-containing plants (48). However, results from several studies using the NPA-containing *Coronilla varia* L. as a forage for cattle indicated that the plant could be safely grazed by cattle (5, 6, 7).

Several studies have been conducted to determine factors that effect the quantity of nitro compound synthesized in the plants. Parker and Williams (39) concluded that higher growing temperatures, 32°C versus 24°C, resulted in higher concentrations of the ether glycoside of NPOH, miserotoxin, in three varieties of *Astragalus* (*A. miser* var. *oblongifolious*, var. *hylophilus*, and var. *serotinus*). They also found that miserotoxin concentrations were reduced when photosynthesis was blocked by removing light for extended periods of time or by disrupting photosynthesis with (2,4,5-trichlorophenoxy)-acetic acid or 2-(2,4,5-trichlorophenoxy)propionic acid. Reduced miserotoxin levels were also observed by Majak et al. (26) when plants were restricted to sunlight. Fertilization did not increase miserotoxin concentrations during the first growing season after application. Parker and Williams (39) applied ammonium nitrate, ammonium sulfate, and potassium nitrate at 56 and 112 kg nitrogen (N) per hectare while Majak and Wikeem (31) applied 100 or 200 kg N urea per hectare. Neither group obtained an increase

in miserotoxin levels after the first year. However, Parker and Williams (39) reported that total nitrite concentrations increased in two varieties of *Astragalus*. Miserotoxin levels increased in *A. miser* var. *serotinus* during the second growing season after the 200 kg urea N per hectare application; however, the possiblities of reduced inter-specific competition, residual N, and favorable moisture conditions contributing to the increased miserotoxin content were not ruled out (31). It has been substantiated that increased moisture favors the accumulation of miserotoxin (26).

The miserotoxin concentrations are highest before flowering (25, 27) and the concentration decreases as the plant matures (25). Accordingly, management practices generally rely on controlled livestock movement as a means of preventing livestock losses (25).

Symptoms

Animals can be either acutely or chronically poisoned with the nitro compounds (12, 13, 30, 54, 56) depending on the amount and the rate of administration of the toxin. Death may occur between 3 to 25 hours in acute cases (12, 13, 21, 52, 53, 58). Chronically poisoned animals may exhibit severe symptoms or die if excited or stressed (12,

13, 21, 56). Animals that have been chronically poisoned may remain affected for more than six weeks after being removed from the poison (14, 56). Symptoms common to both acute and chronic intoxications include labored breathing, increased heart rate, depression, muscular incoordination and knuckling of the fetlocks (12, 13, 14, 21, 30, 53, 56, 58). Other symptoms observed in chronically poisoned animals are frothiness at the nose (13, 21, 30, 53), frequent urinations (14, 53, 56, 58), and weight loss (14, 21, 55). Rats and meadow voles also exhibit a characteristic arching of the back when poisoned with NPA (45).

Nonruminants

Conflicting evidence exists regarding the breakdown of miserotoxin in the monogastric stomach. Mosher et al. (35) demonstrated the production of glucose and NPA from the *in vitro* acid-hydrolysis of miserotoxin with concentrated hydrochloric acid. They concluded that miserotoxin is hydrolized into these metabolites in the monogastric stomach. These results contradict the observations of Williams et al. (58) who reported that the acid-hydrolysis products of miserotoxin were glucose and NPOH. The most recent evidence now suggests that miserotoxin is not degraded to NPA in the stomachs of monogastrics, but that it

is either absorbed as the intact glycoside or passed to the intestinal tract where microbial β-glucosidase activity hydrolyzes the conjugate to NPOH (28). A relatively large dose is required for the latter to occur, however, because miserotoxin is absorbed rapidly from the upper gastrointestinal tract (28).

Monogastric animals may therefore be less susceptible to aliphatic nitro poisoning from plants containing NPOH when the aglycone is conjugated as the glycoside miserotoxin than when unconjugated (27). The intact glycoside was relatively innocuous when orally administered to rats. The LD50 for NPOH was 77 mg/kg body wt compared to the LD50 of greater than 2.5 g/kg body wt for miserotoxin (28). Rabbits are sensitive to poisoning from miserotoxin however, and the toxic effects of miserotoxin appear to be due to nitrite rather than NPOH. This suggests that nitrite is hydrolyzed from miserotoxin without the liberation of NPOH. When rabbits were dosed with extracts from plants containing miserotoxin, they developed acute nitrite poisoning (methemoglobinemia) rather than aliphatic nitro toxicity (58). However, when rabbits were dosed with extracts of the plants containing miserotoxin and then treated with 3 mg/kg of methylene blue as a therapeutic treatment for methemoglobinemia, they did not develop symptoms of toxicity (58). Rabbits dosed with equivalent amounts of NPA and then

treated with methylene blue were not protected from aliphatic nitro poisoning (58). This demonstrates that unconjugated aliphatic nitro compounds are poisonous to rabbits. More recent evidence also suggests that miserotoxin is metabolized to release nitrite in the rabbit gastrointestinal tract without the hydrolysis of the glycosidic bond (27). Intraperitoneal administration of 100 mg/kg body wt miserotoxin to rabbits resulted in high levels of the glycoside in plasma, but the glycoside could not be detected in plasma when 100 mg/kg body wt miserotoxin was given orally (27). Conversely, intraperitoneal administration of the glycoside resulted in 61% lower plasma nitrite concentrations than those found for oral administration of miserotoxin (27). Neither NPA or NPOH were detected in the plasma of the rabbits treated orally or intraperitoneally (27).

Mammals possess sufficient esterase activity in the small intestine to metabolize esters of NPA (27). Therefore, monogastric animals are susceptible to poisoning from plants containing conjugates of NPA. This is emphasized by the recommendation not to feed the NPA-containing legume *Coronilla varia* to monogastric animals (9, 45).

Earlier, Williams and James (52) reported that NPOH-containing plants were more toxic than NPA-containing plants (when reported as per gram of plant material or as mg

nitrite/kg body wt) to 1 week old chicks. Therefore, the general claim that monogastric animals are less susceptible to NPOH than NPA (27) may be inaccurate.

Ruminants

Whereas monogastrics lack β-glucosidase activity in the upper gastrointestinal tract, the microbial population inhabiting the rumen possesses an abundant supply of the enzyme (27). Williams et al. (57) demonstrated that the disappearance of miserotoxin, when incubated *in vitro* with ovine or bovine rumen fluid, coincided with the appearance of NPOH. The ruminal metabolism of miserotoxin proceeds rapidly; they could not detect the glycoside in either ovine or bovine rumen fluid after 4 hours incubation. Furthermore, they did not find NPA as a metabolite of miserotoxin hydrolysis (57). The rapid hydrolysis of miserotoxin to NPOH in rumen fluid has been substantiated by other experiments (29). Esters of NPA are also readily hydrolyzed by ruminants (27). When incubated in rumen fluid, the NPA esters were rapidly metabolized within 4 hours to NPA and presumably glucose (10). There have been no reports of the interconversion of NPA to NPOH or vice versa within the rumen.

The first indication of microbial metabolism of NPA and NPOH came from toxicologicol studies with cattle; however, *in vitro* studies using rumen fluid provided insight about the differential rates in which the two compounds are metabolized. Animals administered milkvetch (*Astragalus miser* var. *oblongifolious*) that contained NPOH or pure NPOH (approximately 60 mg NPOH/kg body wt for both treatments) developed identical symptoms of poisoning (58). However, a heifer dosed with approximately 120 mg NPA/kg body wt did not develop symptoms (58). In a later study, *in vitro* incubations of NPA in rumen fluid resulted in the complete degradation of NPA to unknown products after 20 to 24 hours (10). Furthermore, meadow voles were protected from NPA poisoning when they were given lyophilized rumen fluid that had been incubated with NPA (10). However, meadow voles given a lyophilized rumen fluid control treatment (contained NPA but was not incubated) were not protected from NPA poisoning (10). Some investigators did not detect NPOH degradation in rumen fluid (9, 57). Later, conclusive evidence for microbial degradation of NPOH was obtained by Majak and Clark (22), and they attributed the previous failures to inactive rumen inocula or to insufficient inoculation volumes. Additionally, they found that the *in vitro* rate of microbial NPA degradation was twice the *in vitro* rate of microbial NPOH degradation and they

suggested that this may explain the lower toxicity of NPA, as compared to NPOH, to ruminants (22). In a later study, Majak and Cheng (17) provided further evidence that NPA is degraded faster than NPOH by mixed rumen microorganisms.

Interestingly, when pure cultures of rumen bacteria were tested by Majak and Cheng (17) for their ability to degrade the aliphatic nitro compounds, ten strains were able to degrade NPA and 5 of these strains were also able to degrade NPOH. No strains were found that degraded NPOH only. In a more recent report, Majak and Cheng (18) stated that of 63 strains tested, 19 degraded the aliphatic nitro compounds. The individual strains capable of metabolizing the compounds were not specified in the later study but were identified as belonging to members of *Bacteroides ruminicola*, *Clostridium* sp., *Coprococcus* sp., *Desulfovibrio desulfuricans*, *Lactobacillus* sp., *Megasphaera elsdenii*, *Peptostreptococcus* sp., *Selenomonas ruminantium*, and *Veillonella alcalescens*. *Coprococcus sp. and Megasphera elsdenii* were the most active strains. All of the strains that degraded the aliphatic nitro compounds had the ability to reduce inorganic nitrite; however, the latter occured at a much faster rate (17, 18). This is significant in light of current speculation that nitrite is cleaved from the aliphatic nitro compound during ruminal metabolism (17, 18). Majak and Cheng (17) detected nitrite from resting

cell suspensions of *M. elsdenii* and mixed rumen bacteria incubated with NPOH. The evidence for the reductive cleavage mechanism was not conclusive, however, because the nitrite yield from the resting cells was less than 5% and nitrite was not detected in growing cultures (17). The authors suggested, however, that since the rate of nitrite metabolism was much greater than that of NPOH metabolism the recovery of nitrite would be small.

Alternatively, although there is no published evidence for this with rumen microbes, it could be proposed that the nitro compounds could be reduced *in situ* to their respective amines similar to a mechanism observed by Angermaier and Simon (3) where whole cell and cell free clostridial preparations reduced 2-nitroethanol to 2-amino-ethanol.

As described above, the rates of degradation affect the toxicities of NPA and NPOH to ruminants. The rates of absorption of the compounds may also affect the relative toxicities of the compounds. Majak et al. (29) demonstrated that the rate of NPOH disappearance from rumen was related to the rate of NPA and inorganic nitrite appearance in plasma, suggesting that NPOH is rapidly absorbed and metabolized to NPA and nitrite. The systemic metabolism will be discussed further in a later section; however, the relationship between ruminal disappearance of NPOH and

plasma appearance of nitrite indicates that plasma nitrite concentrations (measured as nitrite or as methemoglobin) are indexes of NPOH absorption. Williams and James (52) determined that in sheep, the maximum plasma methemoglobin concentration reached 26.7% at 3 hours post NPOH ingestion whereas the maximum plasma methemoglobin concentration only reached 1.5% at 5 hours post NPA ingestion. They concluded this indicates that NPA is absorbed more slowly than NPOH (52). In another study, ovine plasma levels of NPA and nitrite were higher after dosing with NPOH than with NPA (40). *In vitro* studies with mixed rumen microorganisms resulted in less than 40% NPOH degradation after 6 hours incubation (17). When this is compared to an average half-life of NPOH within the bovine rumen of 1.24 hours (29), it seems possible that a significant amount of the toxin would be absorbed before ruminal metabolism occurred. While absorption of NPOH is possible in the abomasum and the small intestine, it appears that the reticulo-rumen is the primary site of absorption for the nitro alcohol (40).

If the rates of degradation or the rates of absorption of the aliphatic nitro compounds could be manipulated then it may be possible to protect the animals from poisoning. Significant differences were detected for *in vitro* NPOH degradation rates in rumen fluid obtained from cattle on different diets. Cattle were grazed on three different

native ranges: Kentucky bluegrass (*Poa pratensis*), pinegrass (*Calamagrostis rubescens*), or bluebunch wheatgrass (*Agropyron spicatum*) as well as on orchardgrass or alfalfa pasture (19). Additionally, feedlot rations of alfalfa hay and barley grain or either alfalfa hay or corn silage were assessed (19). The native range diets, especially the Kentucky bluegrass diets, resulted in the highest rates of NPOH degradation (19). Forage quality also affected rates of ruminal NPOH degradation; a fresh stand of orchardgrass pasture (24% crude protein on dry matter basis) supported higher degradation rates than did a ten-year-old stand of orchardgrass pasture (16% crude protein on dry matter basis; 19). Cured orchardgrass hay was reported to be associated with lower rates of NPOH degradation than the fresh orchardgrass pasture; however, there was no significant difference between cured or fresh alfalfa (19). Corn silage rations resulted in the lowest rates of NPOH degradation. Significant differences were not observed between alfalfa hay diets, either with or without grain supplements, which demonstrated that high energy diets alone did not stimulate NPOH degradation (19). In a later experiment, orchardgrass pasture supplemented with molasses resulted in average rates of ruminal NPOH metabolism that were 24% higher than those for orchardgrass pasture alone. However, differences in average NPOH degradation rates were

not significant for a similar comparison between alfalfa supplemented with molasses and alfalfa alone (20). Nevertheless, these results suggest that the ruminal metabolism of NPOH can be manipulated with diet constituents. More recently, when the less toxic nitro compound, nitroethane, was supplemented (20 mg/kg body wt) to cattle, the *in vitro* rate of microbial NPOH was subtantially (4-fold) increased over rates in rumen fluid from cattle receiving unsupplemented diets (20).

As mentioned above, enhancing the microbial metabolism of NPOH under field conditions may reduce the toxicity of plants containing this compound. However, the rapid absorption of NPOH across the rumen epithelium may preclude this strategy. Furthermore, enhancing the microbial metabolism of NPOH may be ineffective when cattle or sheep eat large quantities of NPOH-containing plants or plants containing high amounts of the toxin. For example, while the microbial metabolism of NPA is thought to be adequate to detoxify this compound in most circumstances, an incident of poisoning has been reported when the NPA-containing *Indigofera endecaphylla* Jacq. (creeping indigo) comprised nearly 50% of the forage diet (38). Additional poisonings due to high doses of other NPA-containing plants have also been reported (55). Alternatively, mention has been made of the

utilization of β-glucosidase inhibitors which would presumably render the intact glycoside innocuous (27).

Systemic Metabolism

Although elevated methemoglobin levels have been used to estimate the rate of absorption of NPA and NPOH and as an index of intoxication (52), methemoglobinemia is generally not the cause of death in acute cases (32, 52). For instance, when methylene blue (3 mg/kg body wt) was given to a milkvetch-poisoned steer as a theraputic treatment against methemoglobinemia, the animal still died of milkvetch poisoning (58). Furthermore, acute methemoglobinenemia is generally associated with methemoglobin concentrations that exceed 80% but the methemoglobin levels are generally less than 6.5% for cattle chronically poisoned by NPA or NPOH (21, 52, 53). Acutely poisoned cattle have plasma methemoglobin concentrations between 20% to 30% within a few hours after being poisoned by the toxins (32, 52, 53, 58).

Once absorbed, the NPOH is converted to NPA by alcohol dehydrogenase (4, 29, 37, 40) and the nitro acid is thought to block the tricarboxcylic acid cycle by inactivation of succinate dehydrogenase (1, 8, 11). It has been postulated that NPOH is enzymatically converted to 3-nitropropion-aldehyde, which then degenerates spontaneously to cytotoxic

acrolein and inorganic nitrite (2). However, the efficient oxidation of NPOH to 3-nitropropionaldehyde and then to NPA *in vivo* by alcohol dehydrogenase without the formation of acrolein has been demonstrated (4). That the later scheme is operative *in vivo* is supported by evidence suggesting that the conversion of NPOH to NPA preceeds the liberation of nitrite (4, 37).

Two mechanisms have been proposed for the inactivation of succinate dehydrogenase by NPA. In one scheme, the nitronate ion of NPA binds irreversibly to the flavin, forming a covalently bound adduct (1). In another scheme, the NPA reacts with succinate dehydrogenase (8). The latter proposal was presented by Coles et al. (8) who showed that the inactivaction of succinate dehydrogenase by NPA essentially eliminated the enzymes capacity to bind to ^{14}C-oxalacetate, suggesting that inactivation of the enzyme occurred at the active site. Additionally, succinate dehydrogenase was rapidly and irreversibly inactivated when 3-nitro-acrylate was added to the reaction mixture. Therefore, this mechanism proceeds with succinate dehydrogenase oxidizing NPA to 3-nitroacrylate which then binds to the enzyme (8).

The toxicity of NPA may not be limited to inactivation of succinate dehydrogenase. Various investigators have suggested that NPA also affects the activities of fumarase

(41), isocitrate lyase (43), catalase (42), and rat brain monoamine oxidase (33). According to Gustine (9) however, the inhibition constants of the later three enzymes were too small to be physiologically significant. Additionally, when rats and sheep were treated with timber milkvetch, significant increases of plasma serum glutamate oxaloacetate transaminase and serum isocitrate dehydrogenase levels were found (34, 35). They concluded that the increases of these enzymes were indications of liver and kidney necrosis.

Analytical Methods

The most common methods of detecting and measuring aliphatic nitro compounds rely on either colorimetric analysis or high performance liquid chromatography (HPLC). Two colorimetric procedures have been used. One procedure is an indirect method that is based on various adaptations of the Griess-Ilosvay assay for nitrous acid (15). The nitro compounds undergo an alkaline treatment to release the nitrite ion which is then measured after diazotization of sulfanilic acid and coupling with 1-naphthylamine (15). This procedure is not recommended because the extended alkalization of NPOH or miserotoxin results in poor yields of nitrite (15, 21). The second colorimetric procedure is based on the reaction of aromatic diazonium salts

(diazotized p-nitroaniline) with the nitronate ion (15). The reaction is restricted to primary and secondary aliphatic nitro compounds that possess at least one acidic alpha hydrogen (15).

Reverse-phase HPLC has been used to detect and quantify NPA and NPOH in plasma, urine, and rumen fluid supernatants (21, 24, 36). However, for NPA determination the rumen fluid supernate should be acidified and extracted with ethyl acetate prior to HPLC in order to achieve adequate resolution (21). The column consists of monomeric octadecylsilane on 5 μm silica, and the samples are eluted isocratically with 0.15% orthophosphoric acid (pH 2) (21, 24, 36).

Although gas chromatography (GC) procedures have been developed for measuring NPOH, the glass columns (5% Carbowax 20 M on Chromosorb W) deteriorated rapidly when injected with untreated gastric samples (21). Additional problems were encountered when the digestive fluids were pretreated before being applied to the GC. For instance, losses of NPOH occurred when the samples were pretreated (21).

Nitrite measurement has been accomplished by the method of Schneider and Yeary (44). Their method is a modification of the procedure proposed by the Association of Official Analytical Chemists in that it uses N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) in place of the highly

carcinogenic 1-naphthylamine (44). Briefly, the procedure involves combining the sample with sulfanilamide reagent (2% sulfanilamide in 4.25% v/v phosphoric acid) and 5% NEDA solution; absorbance is then measured at 540 nm (44).

SECTION 1. METABOLISM OF THE PLANT TOXINS NITROPROPIONIC ACID AND NITROPROPANOL BY RUMEN MICROORGANISMS

Metabolism of the plant toxins nitropropionic acid and nitropropanol by rumen microorganisms

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ABSTRACT

Nitropropionic acid (NPA) and nitropropanol (NPOH) are plant toxins that reduce weight gains and can result in death of grazing ruminants. Ruminal microbes can degrade these toxins but the mechanism of degradation and factors that affect degradation remain unclear. Tests with ovine and bovine ruminal microbes indicated that the nitro groups of NPA and NPOH were reduced to the respective amines. In the case of NPA, quantities of β-alanine that were recovered accounted for less than 50% of the NPA metabolized. However, we discovered that β-alanine is further metabolized by the microbes. The production of 3-amino-1-propanol accounted for >83% of the >3 μmole NPOH/ml metabolized after 8 h by the ruminal microbes. Nitrite accounted for <1% of the NPA or NPOH metabolized from these suspensions. When ferrous and sulfide ions were added to cell suspensions a 3-fold increase in the rates of NPOH reduction and 3-amino-1-propanol production were obtained. These results suggest that a hydrogenase/ferredoxin system may be operating in the reduction and that ferrous and sulfide ions may act by protecting ferredoxin from inhibition by nitrite.

INTRODUCTION

The nitro-toxins, 3-nitro-1-propionic acid (NPA) and 3-nitro-1-propanol (NPOH), are responsible for poisoning thousands of sheep and cattle each year (22). More than 450 species of the legume *Astragalus* synthesize either NPA or NPOH (22, 23, 24). Additionally, NPA is synthesized by certain species of *Coronilla, Indigofera*, and *Lotus*; these genera are also legumes (15). In plants, NPA commonly exists as glucose esters whereas NPOH exists as ether glycosides. These conjugates are rapidly hydroyzed within the rumen to glucose and NPA or NPOH (15). Ruminal microorganisms metabolize NPA rapidly and NPOH slowly (7, 10, 14). This may be why NPOH is more toxic than NPA to ruminants (14, 15).

Some researchers have attempted to manipulate the rumen environment to enhance the rates of microbial NPOH metabolism and thus reduce its toxicity (10, 11, 12, 13, 14). For instance, ruminal microbes collected from cattle receiving 20 mg/kg nitroethane, a nontoxic analog of NPOH, as a diet supplement metabolized NPOH 4X faster than microbes collected from cattle not receiving the supplement (13). Additionally, it has been shown that the rates of NPOH metabolism in the rumen are influenced by the type of forage fed (12). While these observations demonstrate that

the rumen environment can be manipulated to enhance the microbial capacity to detoxify NPOH, the reasons for these effects are not well understood.

Correlations exist between the metabolic capacity for NPA and NPOH degradation and nitrite reduction using pure strains of rumen bacteria. Additionally, nitrite was detected in resting cell cultures of rumen microorganisms incubated with NPA or NPOH (10). These results suggested that NPA and NPOH metabolism involved a reductive cleavage of the nitrite group from the carbon chain (10). The evidence for this mechanism was not conclusive, however, because the nitrite yield was less than 5% in the resting cell cultures.

An understanding of the mechanisms by which NPA and NPOH are metabolized may eventually lead to the development of practical strategies designed to prevent poisonings. Therefore, our objectives were to elucidate the metabolic steps involved in microbial NPA and NPOH degradation and factors that influence their metabolism. Our observations revealed that a reduction of NPA and NPOH to β-alanine and 3-amino-1-propanol, respectively, was the primary mechanism involved. We present evidence supporting this conclusion and results from initial studies designed to elucidate factors affecting the reaction.

MATERIALS AND METHODS

Source and Preparation of Mixed Rumen Microorganisms

Bovine rumen microorganisms were obtained by collecting rumen fluid from a fistulated cow receiving an alfalfa hay:chopped corn diet (9:1). Ovine rumen microorganisms were collected from a fistulated sheep receiving a similar diet. Each animal had access to salt and trace mineral supplements. Unless otherwise specified in the text, rumen fluid was collected approximately 1 to 2 h after the animals had been fed. Preliminary studies indicated that rumen fluid samples collected at these times had faster rates of NPA or NPOH metabolism than samples collected 5 or 24 h after feeding. The rumen fluid was strained through 2 layers of cheese cloth into insulated containers which were capped and immediately returned to the laboratory.

Unless otherwise specified in the text, mixed rumen microorganisms were either incubated as the cells in whole rumen fluid or as cells resuspended in anaerobic dilution solution (andil; 4). Resuspended rumen microorganisms were prepared by centrifuging whole rumen fluid (10 min at 18,000 x g), discarding the supernatant fluid and resuspending the cells with a volume of andil either equal to, or 1/3 of the

intial volume of rumen fluid. Oak Ridge Centrifuge bottles (Du Pont, Wilmington, DE) fitted with o-rings and sealing assemblies which permit use of techniques that exclude air were used. Unless otherwise stated, CO_2 was used as the gas phase. After the cells had been resuspended, 10-ml aliquots were distributed to 18 x 150 mm culture tubes and incubated at 39°C during intervals indicated in the text. Test substrates were added as small volumes (<0.5 ml) of concentrated stock solutions. All procedures were performed under anaerobic conditions (3, 8).

Effect of NPA and NPOH on Growth of Rumen Bacteria

Serial dilutions of rumen contents were inoculated into roll tubes (18 x 150 mm) containing 6 ml of medium 98-5 (5) modified to contain 0.025% xylose, 0.4% Na_2CO_3 and 6/7 the normal volume of H_2O. Additionally, the medium was prepared under CO_2 rather than N_2. Immediately before inoculation, 1 ml of anaerobic solutions of NPA or NPOH in H_2O (pH 6.7-6.8) were added to achieve final concentrations of 0, 4.2, 21, or 42 mM. Triplicate cultures of each concentration and each dilution from 10^{-7} to 10^{-9} were incubated. Colony forming units (CFU) were counted after 7 d with the aid of a dissecting microscope.

Analytical Methods

Subsamples (1 or 1.5 ml) were removed at intervals from the incubations and were clarified by centrifugation for 10 min at 16,000 x g. The supernatants were stored at -25°C until analysis. NPA and NPOH were measured by using a modification of the colorimetric method of Majak et al. (12). Samples or standards (50 μl) were diluted with 5 ml of H_2O followed by the addition of 100 μl of 0.65 M NaOH and 100 μl diazotized paranitroaniline. Absorbance was read at 405 nm. Nitrite was measured by the method of Schneider and Yeary (21) at 540 nm. Short-chain organic acids were measured by gas chromotography of their butyl esters (20) as modified by Allison et al. (1); the detection limit was <0.10 μmole/ml. Alcohols were measured by gas chromotography.

High performance liquid chromotography (HPLC, System Gold, Beckman Instruments, San Ramon, CA) of the dabsylated amino compounds was accomplished at 44°C by using a Brownlee spheri-5, RP-18 column (220 x 4.6 mm, Applied Biosystems, Inc., San Jose, CA). Absorbance was measured with a diode array spectrophotometer at 436 nm. Integrated peak area was used to quantify the amino compounds (detection limit, <0.1 μmole/ml); norleucine (5 mM) was an internal standard. The mobile phase consisted of a programmed gradient of sodium

acetate with 4% N,N-dimethylformamide buffer (solvent A) and acetonitrile (solvent B). Solvent A was prepared by combining 2.95 ml glacial acetic acid and 1 l H_2O and then adjusting the pH of this solution to 6.5 with 30% NaOH. Dimethylformamide (80 ml) was added, and the volume was adjusted to 2 l with H_2O. The solvents were filtered through 0.2-μm nylon 66 membrane filters and degassed prior to use. The gradient, with a flow rate of 1 ml/min, was (solvent A) 90%, 65%, 45%, 45%, 15%, 90%, and 90% at 0.0, 0.1, 30.0, 42.0, 44.0, 46.0, and 62.0 min, respectively, with solvent B making up the remaining percentages.

Samples or standards (20 μl) plus 10 μl 5 mM norleucine stock solution were mixed in 13 x 100 mm screw-cap tubes with 80 μl 50 mM sodium bicarbonate and 160 μl dabsyl chloride reagent (4 μmole/ml in acetonitrile). The tubes were sealed with teflon-lined caps and heated at 70°C for 10 min. After cooling, the mixture was diluted with 2.0 ml of 50 mM phosphate buffer (pH 7):ethanol (1:1), mixed and filtered through a 0.22 μm Millipore filter. The injection volume was 50 μl.

Ascending thin layer chromotography (TLC) was performed on preactivated (110°C for 30 min) Avicel cellulose plates (20 x 20) using butanol:acetic acid:H_2O (4:1:2.2). The amino compounds were detected by spraying with ninhydrin reagent (18).

Chemicals and Reagents

Nitropropionic acid (98% pure) and 3-amino-1-propanol were purchased from Aldrich (Milwaukee, WI). Nitropropanol (90% pure) was purchased from Chem-Biochem Research, Inc. (Salt Lake City, UT). Dabsyl chloride (4-dimethylaminoazobenzene-4'-sulfonyl chloride) and β-alanine were purchased from Sigma (St. Louis, MO). Organic solvents were either HPLC grade or distilled before use. All other chemicals were of analytical or technical grade.

RESULTS

Effect of NPA and NPOH on Growth of Rumen Microorganisms

To determine if NPA or NPOH inhibited the growth of bovine ruminal bacteria we counted the number of CFU appearing in anaerobic roll tubes incubated with the nitro-toxins. After 7 days of incubation, colony counts were 90, 30, and 24% of control counts (medium without additions, $1.8 \pm 0.5 \times 10^9$ CFU/g wet wt) for media containing 4.2, 21, or 42 mM NPA, respectively. Colony counts were 81, 32, and 10% of control counts for media with 4.2, 21, or 42 mM NPOH, respectively.

Determination of Metabolic Products

The mean rates of NPA and NPOH disappearance after 6 h incubation of bovine rumen fluid samples collected and incubated on 3 different days with 4.2 mM NPA or NPOH were 0.43 ± 0.14 and 0.10 ± 0.09 μmole/ml/h, respectively. In order to enhance detection of the reaction products, microbial cells at 3X concentration were resuspended in andil and incubated with 12.6 mM NPA, 12.6 mM NPOH or without either compound. The rates of NPA and NPOH

disappearance after 6 h for these cell suspensions were 0.92 ± 0.14 and 0.42 ± 0.15 μmole/ml/h, respectively. The values are mean ± SD from duplicate incubations of microbes from 1 collection.

The production of 6.3 μmole propionate/ml by concentrated suspensions of bovine microbes incubated 22 h with 12.6 mM NPA was slightly higher than that by similar suspensions incubated without NPA (5.2 μmole/ml), but this difference accounted for <10% of the >12 μmole NPA/ml metabolized. No propanol was detected when concentrated suspensions of bovine microbes were incubated with NPOH, even though 10.4 μmole NPOH/ml had been metabolized after 22 h. In contrast, even though >7 μmole NPA/ml had been degraded after 8 h by concentrated suspenions of ovine rumen microbes, the amount of propionate produced by these suspensions (9.9 μmole/ml) was less than that produced by suspensions incubated without NPA (10.8 μmole/ml). However, when concentrated ovine microbes were incubated 8 h with 12.6 mM NPOH, 0.18 μmole propanol/ml was produced; this accounted for 5% of the 3.5 μmole NPOH/ml metabolized. We detected 0.02 and 0.04 μmole nitrite/ml after 5 h and 0.01 and 0.02 μmole nitrite/ml after 8 h incubation when the ovine suspensions were incubated with 12.6 mM NPA or NPOH, respectively. However, the amount of nitrite detected

accounted for <1% of the amount of NPA or NPOH metabolized. We did not measure nitrite in the bovine suspensions.

When supernatant fluids from the above incubations were derivitized with dabsyl chloride and then analyzed by HPLC, we obtained evidence that the nitro groups on both NPA and NPOH had not been split off but had been reduced *in situ* to their respective amines (Figure 1). Thus, β-alanine was produced in incubations with NPA (C, Figure 1) and 3-amino-1-propanol was produced in incubations with NPOH (B, Figure 1). Levels of ammonia that accumulated in incubations with NPA and NPOH were similar to accumulations in incubations without added NPA or NPOH. Similar elution profiles were obtained using ovine microbes. When subsamples from the ovine incubations were spiked with known β-alanine or 3-amino-1-propanol and analyzed for the dabsyl derivatives, we observed increases in the corresponding peak areas.

The amounts of β-alanine and 3-amino-1-propanol detected from the ovine suspensions after 8 h of incubation with NPA or NPOH were 3.7 μmole/ml and 3.0 μmole/ml, respectively. The disappearance of NPA and NPOH and the appearance of β-alanine and 3-amino-1-propanol from the samples taken from incubations of bovine microbes are shown in Figures 2 and 3. The appearance of β-alanine was not stoichiometric with the disappearance of NPA (Figure 2); however, the appearance of 3-amino-1-propanol coincided well with the

disappearance of NPOH (Figure 3). The absence of β-alanine at 22 h suggested that this product was further metabolized by the rumen microbes. This was confirmed in another experiment (data not shown) in which over 70% of added β-alanine (12.6 mM) disappeared from similar suspensions of rumen microbes within 22 h. We did not observe the disappearance of 3-amino-1-propanol when rumen microorganisms were similarly incubated with the amino-alcohol. TLC analysis from the bovine incubations provided further evidence that the nitro-compounds were metabolized to the amino-compounds. Samples from incubations with NPA or NPOH had ninhydrin-reactive compounds that migrated with known β-alanine or 3-amino-1-propanol (RF 0.48 and 0.58, respectively) but these were not seen in samples from control incubations (data not shown).

The reduction of NPA to β-alanine and of NPOH to 3-amino-1-propanol by ruminal microbes was also observed in an environment more closely representing the rumen (whole rumen fluid) than that of the concentrated cell suspensions (Appendix).

Effect of Hydrogen Donor Addition on NPOH Metabolism

Several experiments were conducted to test the effects of adding various potential hydrogen donors on NPOH

metabolism. The addition of 6.5 mM citrate, formate, glucose, glycerol, lactate, mannitol, pyruvate, succinate, or xylose to concentrated suspensions of bovine microbes did not result in increased rates of NPOH metabolism after 6 h incubation as compared to suspensions incubated without the potential donors (data not shown). We also tested the effect of replacing the CO_2 gas phase with a 1:1 mixture of H_2:CO_2. In one experiment, the rate of NPOH metabolism for suspensions incubated with H_2:CO_2 were enhanced 28% compared to controls incubated under CO_2 (1.02 $\pm$ 0.01 and 0.80 $\pm$ 0.16, respectively). However, enhancement of NPOH degradation by H_2 varied. In a separate experiment, 3X concentrated bovine microbes were incubated with NPOH under 100% CO_2, 100% N_2, 100% Ar, 100% CO or H_2:CO_2 (1:1). The cultures were incubated 12 h in crimp-top tubes under approximately 202 kPa (2 atm) CO_2, N_2, Ar, approximately 180 kPa (1.8 atm) CO or approximately 283 kPa (2.8 atm) H_2 (mix). The rates of NPOH metabolism obtained from triplicate cultures were 0.47 $\pm$ 0.02, 0.67 $\pm$ 0.01, 0.70 $\pm$ 0.02, 0.19 $\pm$ 0.03 and 0.45 $\pm$ 0.03 μmole/ml/h, respectively.

When bovine rumen microorganisms were washed to reduce endogenous energy yielding substrates, no increase was observed in the rate of NPOH metabolism due to the addition of 6.5 mM citrate, galactose, glucose, glycerol, lactate, or mannitol (data not shown). However, incubation with

H_2:CO_2 (1:1) increased the rate of NPOH metabolism over controls by 76% (0.30 ± 0.10 and 0.17 ± 0.09, respectively). The washing treatment decreased the NPOH-degrading activity of incubations with and without added hydrogen donors. Initial pH values of all the treatments ranged from 6.31 to 6.47 whereas the pH ranged from 6.34 to 6.51 between treatments at 6 h. We consider the effect of pH within this narrow range to be minimal during the 6 h incubation.

Effect of Ferrous and Sulfide Ions on NPOH Metabolism

A 3-fold increase in the rate of NPOH disappearance was obtained when both ferrous sulfate (20 mM) and sodium sulfide (2.5 mM) were added to suspensions of mixed rumen microbes (Table 1). The addition of only ferrous sulfate at 10 mM or 20 mM also increased the rate of NPOH metabolism; however, the increase was not as great as when sodium sulfide was included. The addition of sodium sulfide without ferrous sulfate had little effect on NPOH metabolism (Table 1). Ferrous sulfate (20 mM) plus sodium sulfide (2.5 mM) also resulted in a 3-fold increase in the rate of end product (3-amino-1-propanol) formation (Figure 4). NPOH metabolism did not occur when autoclaved rumen microbial preparations were similarly incubated with ferrous sulfate plus sodium sulfide. We also found that ferrous sulfate

plus sodium sulfide additions increased the rate of NPOH metabolism in an environment more closely representing the rumen than is the case for the above suspensions of cells. Thus, the addition of ferrous sulfate and sodium sulfide to whole rumen fluid increased the rate of NPOH disappearance; however, these supplements had little effect on NPA metabolism (Figure 5).

DISCUSSION

Our observations that mixed populations of ruminal microbes incubated *in vitro* metabolize NPA more rapidly than NPOH are in agreement with those reported earlier (10, 14); however, the reason for this rate differential is not known. We obtained evidence that the principle metabolic pathway for the microbial metabolism of NPA and NPOH involved an *in situ* reduction, producing β-alanine and 3-amino-1-propanol, respectively. In the case of NPA, β-alanine accounted for 36% and 50% of the NPA metabolized by bovine and ovine ruminal microbes, respectively. Since Looper et al. (9) demonstrated, and we confirmed here, that β-alanine is further metabolized by ruminal microbes, it is not possible to quantify β-alanine formation from measurements of β-alanine concentrations. Of the NPOH metabolized by bovine and ovine ruminal microbes, over 87% and 85%, respectively, was recovered as 3-amino-1-propanol. Further research is needed to determine if 3-amino-1-propanol is metabolized within the rumen, but our data from *in vitro* incubations indicated that 3-amino-1-propanol was metabolized slowly, if at all. We are currently investigating potential rate-limiting processes involved in the reduction which may lead to the development of stategies

designed to drive the reaction; thereby, increasing the microbial detoxification capacity. Results from initial studies are presented in this paper.

In view of the considerable body of knowledge about the reduction of nitrocompounds by anaerobic microbes (6, 16, 17, 19) our findings that indicate rumen microbes reduce NPA and NPOH are not unexpected. Angermaier and Simon (2) reported that 2-nitroethanol was reduced to 2-aminoethanol by clostridial whole cell preparations, crude extracts and by a combination of purified hydrogenase and ferredoxin. According to Angermaier and Simon (2), a nonspecfic hydrogenase/ferredoxin system, which was inactivated by nitrite and subsequently reactivated by the inclusion of ferrous and sulfide ions, was involved in the reduction of the nitro alkane.

A hydrogenase/ferredoxin reducing system similar to that functioning in clostridia is suggested by our findings that CO, a known hydrogenase inhibitor, depressed the rate of NPOH metabolism and our observations that H_2, in two experiments, enhanced the rate of NPOH metabolism. No increase in the rate of NPOH metabolism due to H_2 was observed in an experiment where the effects of different gas phases were compared. However, the pH may have been decreased further during incubations under CO_2 (or H_2 mixed 1:1 with CO_2) than incubations under N_2 or argon

which might have resulted in depressed rates. If a hydrogenase/ferredoxin system is operating in the reduction of NPOH, it may explain our inability to stimulate NPOH metabolism by supplying alternative hydrogen donors to the cell suspensions. Majak et al. (12) reported that rates of NPOH metabolism were not significantly different when ruminal microbes were incubated in rumen fluid collected from cattle receiving alfalfa hay with or without barley supplementation. Presumably, the barley supplementation would have provided a broader range of reducing equivalents than we used. Additionally, evidence obtained using whole rumen fluid incubations supplemented with complex mixtures of carbohydrates suggested that NPA reduction was not increased when a wider range of reducing equivelants were available (Appendix). However, comparing differences between changes in rates of microbial NPOH and NPA metabolism may not be appropriate. It is possible that the reducing system may be limited by some other mechanism, such as nitrite inhibition, and this may mask effects of the hydrogen donors. Additional research is needed to elucidate this possibility.

Angermaier and Simon (2) detected as one of the reaction intermediates of 2-nitroethanol metabolism, an unstable radical which released a small amount of nitrite (<4% yield) which was responsible for the subsequent

inactivation of ferredoxin needed for the reduction. We detected small quantities of nitrite in the reaction mixture when ovine rumen microbes were incubated with NPA or NPOH. Additionally, our observations of a small amount of propanol produced by suspensions of ovine microbes incubated with NPOH and of the differences in propionate production by bovine rumen microbes incubated with and without NPA support the concept that some cleavage may have indeed occurred. Majak and Cheng (10) also detected the occurrence of nitrite during incubations of ruminal microbes with NPA or NPOH. They suggested the nitrite was subsequently reduced to ammonia by ruminal microbes, resulting in its detoxification. Conversely, an inactivation of ferredoxin in our system by nitrite is suggested by the 3-fold stimulation in rates of NPOH metabolism and 3-amino-1-propanol formation when ferrous and sulfide ions were supplied. Angermaier and Simon (2) proposed that ferrous and sulfide ions formed biologically active clusters that reconstituted inactivated ferredoxin or apoferredoxin to a functionally active ferredoxin. If radical intermediates are formed during the reduction of NPA and NPOH, their relative stabilities may be involved in the differential rates at which NPA and NPOH are metabolized.

While it has previously been demonstrated that the rumen environment can be manipulated to enhance the rate of NPOH metabolism, the factors involved are not well defined. For instance, higher rates of microbial NPOH metabolism were associated with Kentucky bluegrass than with the following forages, which are listed in descending order in regards to associated rates of NPOH metabolism: pinegrass>bluebunch wheatgrass>orchard grass>alfalfa (13). In view of our results from the ferrous and sulfide supplements, the diet effects could possibly be related to the content of these minerals within the forage. Presumably, ferrous iron would be the limiting mineral because sulfate, which is rapidly reduced to sulfide by ruminal microbes, has been supplemented to cattle diets with no resultant increase in NPOH degradation (13). Also, our results with sulfide additions to *in vitro* incubations indicate that sulfide by itself does not result in increased rates of NPOH degradation (Table 1). Additional research will determine the practicality of supplying these ions to ruminant diets as a means of preventing poisonings. Our results from preliminary *in vitro* experiments suggest that the ferrous and sulfide ion supplements enhance NPOH, but not NPA, metabolism by microbes as they occur in whole rumen fluid (Figure 5) as well as by concentrated suspensions of cells.

Potentially, optimal concentrations and forms in which the ions are presented may allow for sufficient microbial detoxification of NPOH to protect animals from poisonings.

Although Majak and Cheng (10, 11) have shown that representatives from several species of ruminal bacteria are able to metabolize NPA and NPOH, little has been reported on the effects of these compounds on the growth of ruminal bacteria. In this regard, we assessed the effects of 4.2, 21, or 42 mM NPA or NPOH on the growth of ruminal bacteria appearing in anaerobic roll tubes. Most of the ruminal bacteria grew in the presence of 4.2 mM NPA or NPOH and some could tolerate high levels of the nitro-toxins. The lower concentration may be near that which might be expected in the rumen of animals grazing a milkvetch range ($\leq$5 mM; assuming a rumen volume of 60 l x an estimated 500 kg animal x 20-60 mg NPOH/kg body wt intoxicating dose; 15). Of the 10% or 20% of the population that were inhibited by 4.2 mM NPA or NPOH, respectively, it is not known if these would be critical for optimal rumen function.

Previous work investigating the microbial metabolism of the nitro-toxins, particularily NPOH, in whole rumen fluid has been hampered because of the relatively low rates at which these compounds are degraded. Our results provide insight into some of the basic biological processes involved

in the metabolism of these compounds. Determination of the applicability of these results *in vivo* await further investigation.

ACKNOWLEDGMENTS

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Table 1. Effects of ferrous sulfate, sodium sulfide, or a combination of these compounds on nitropropanol metabolism by bovine rumen microorganisms[a]

Rate of nitropropanol disappearance (μmole/ml/h)[b]			
	Concentration of added substrate		
$FeSO_4$[c] (mM)	Na_2S[c] (mM)		
	0.0	2.5	5.0
0.0	0.45 ± 0.12	0.52 ± 0.03	0.51 ± 0.04
10.0	0.70 ± 0.25	ND[d]	1.42 ± 0.09
20.0	1.10 ± 0.15	1.66 ± 0.07	1.45 ± 0.20
40.0	0.53 ± 0.09	ND	0.92 ± 0.10

[a]Concentrated (3X) mixed bovine microbial suspensions were incubated 6 h in anaerobic dilution solution initially containing 12.6 mM nitropropanol.

[b]Mean ± SD from triplicate cultures.

[c]$FeSO_4 \cdot 9H_2O$ or $Na_2S \cdot 9H_2$.

[d]ND, not determined.

Figure 1. HPLC chromatograms of dabsylated amino compounds identified from suspensions of mixed bovine ruminal microorganisms incubated 22 h without either nitropropanol (NPOH) or nitropropionic acid (NPA) (A), 22 h with NPOH (B), or 10 h with NPA (C); peaks are identified in the figure

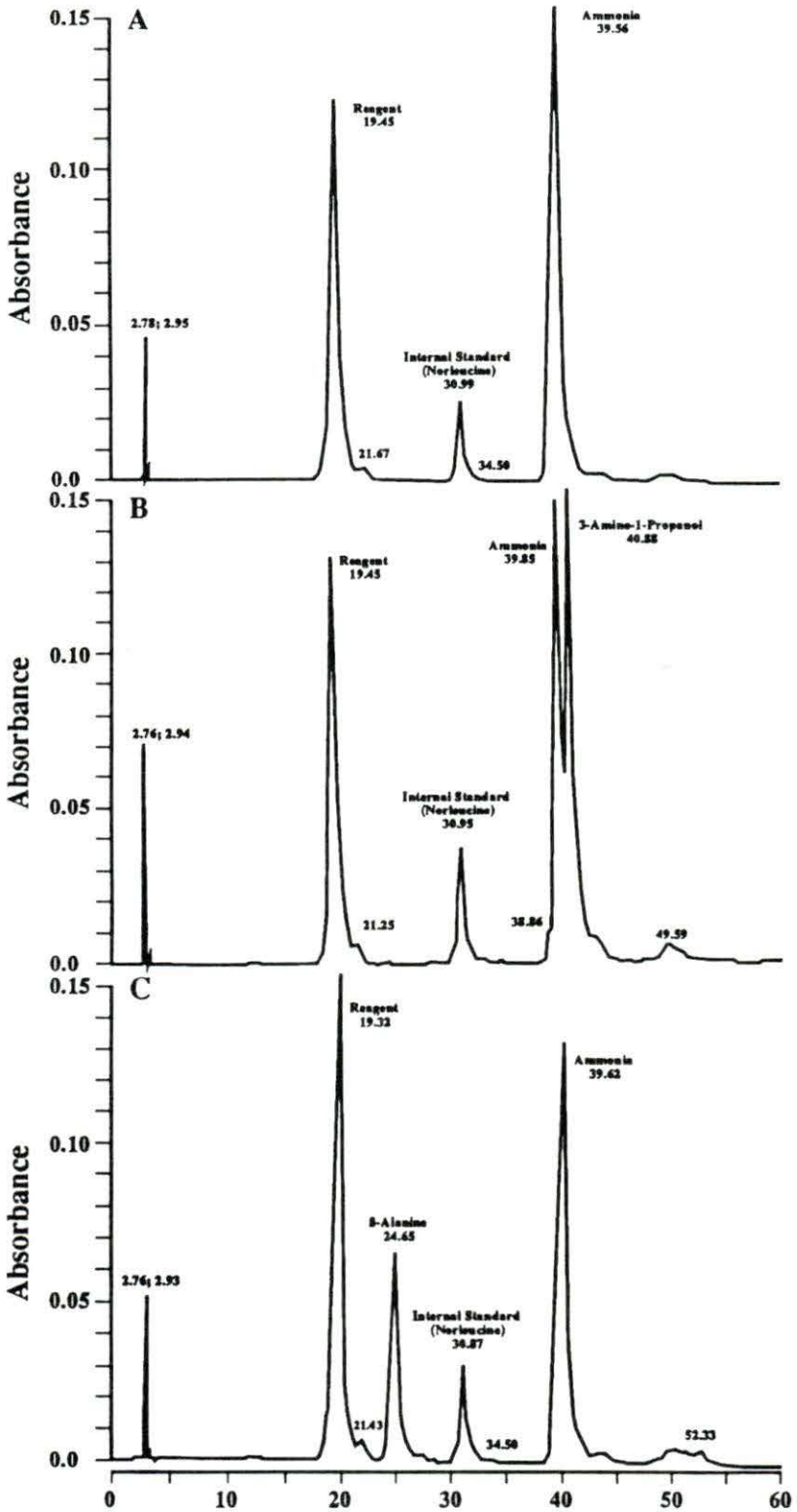

A
Absorbance
0.15
0.10
0.05
0.0
Ammonia
39.56
Reagent
19.45
2.78; 2.95
Internal Standard
(Norleucine)
30.99
21.67
34.50
B
Absorbance
0.15
0.10
0.05
0.0
3-Amino-1-Propanol
40.88
Ammonia
39.85
Reagent
19.45
2.76; 2.94
Internal Standard
(Norleucine)
30.95
21.25
38.86
49.59
C
Absorbance
0.15
0.10
0.05
0.0
Reagent
19.32
Ammonia
39.62
β-Alanine
24.65
2.76; 2.93
Internal Standard
(Norleucine)
30.87
21.43
34.50
52.33
0
10
20
30
40
50
60

Figure 2. Changes in nitropropionic acid (circles) and β-alanine (squares) concentrations during incubation of concentrated (3X) bovine rumen microbial suspensions with 12.6 mM nitropropionic acid; microbes were harvested from rumen fluid collected approximately 22 h after feeding

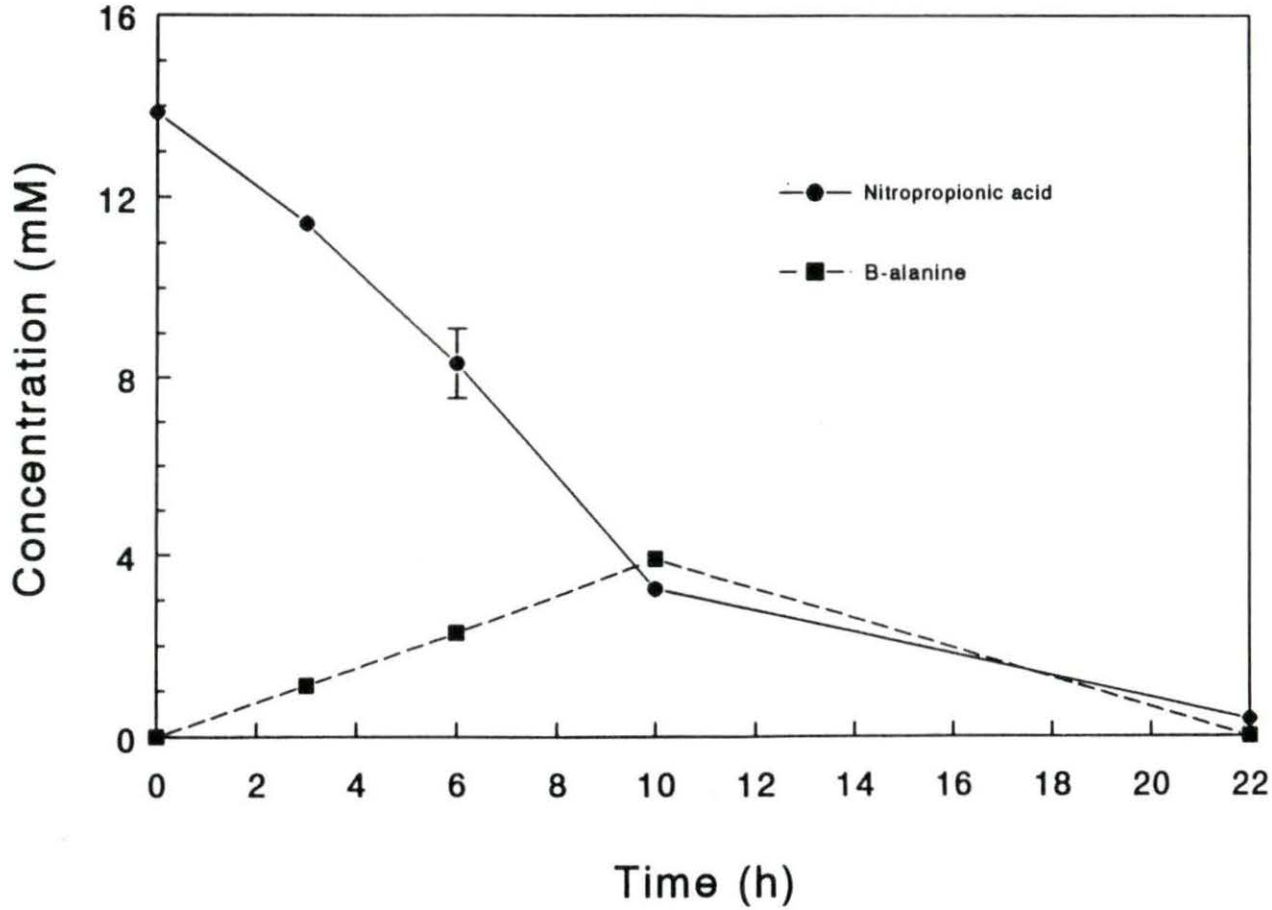

16
12
8
4
0
Concentration (mM)
Nitropropionic acid
B-alanine
0
2
4
6
8
10
12
14
16
18
20
22
Time (h)

Figure 3. Changes in nitropropanol (circles) and 3-amino-1-propanol (squares) concentrations during incubation of concentrated (3X) bovine rumen microbial suspensions with 12.6 mM nitropropanol; microbes were harvested from rumen fluid collected approximately 22 h after feeding

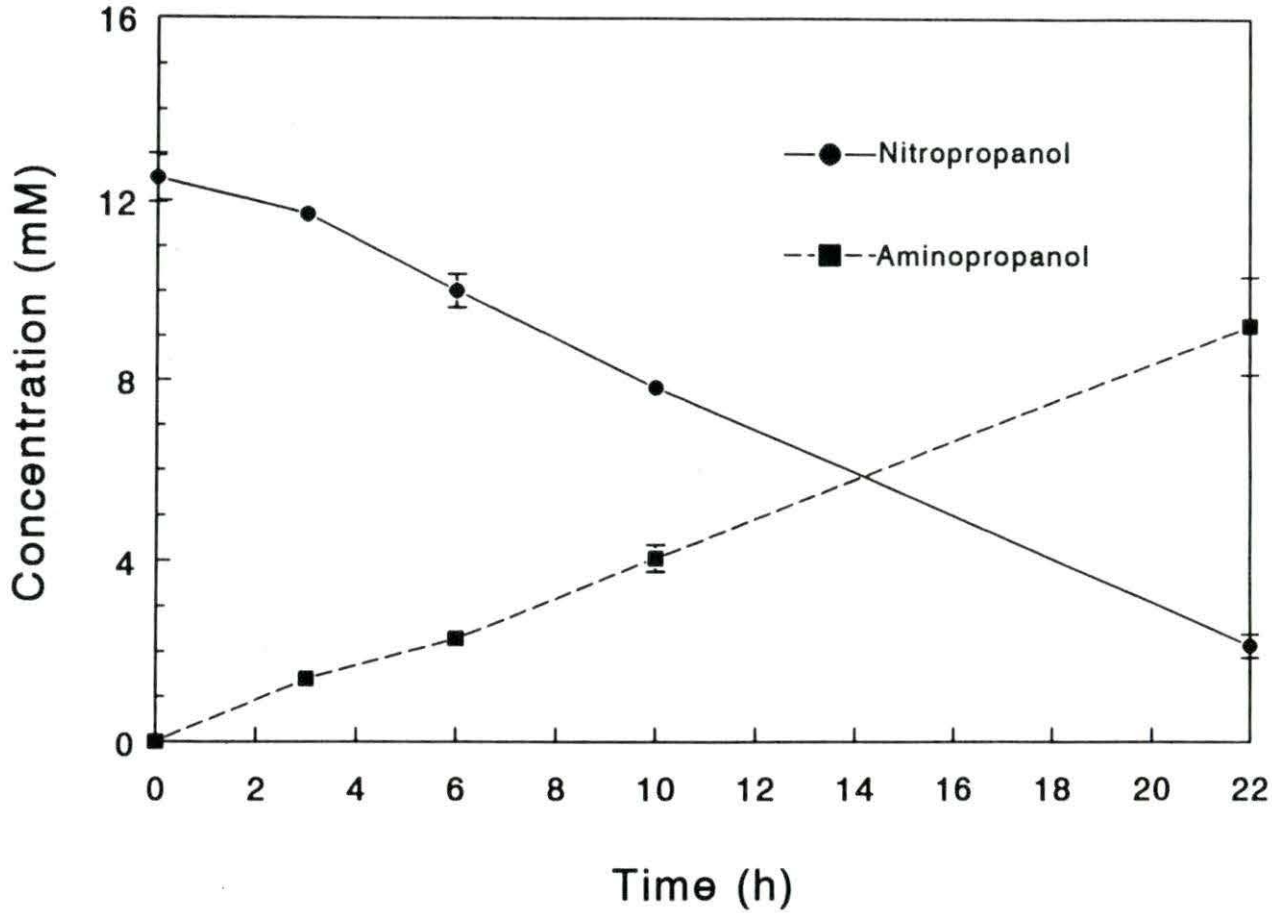

16
12
8
4
0
Concentration (mM)
Nitropropanol
Aminopropanol
0
2
4
6
8
10
12
14
16
18
20
22
Time (h)

Figure 4. Changes in nitropropanol (circles) and 3-amino-1-propanol (squares) concentrations during incubations of concentrated (3X) bovine rumen microbial suspensions in the presence of 12.6 mM NPOH without (open legends) or with (closed legends) ferrous sulfate (20 mM) plus sodium sulfide (2.5 mM) supplement

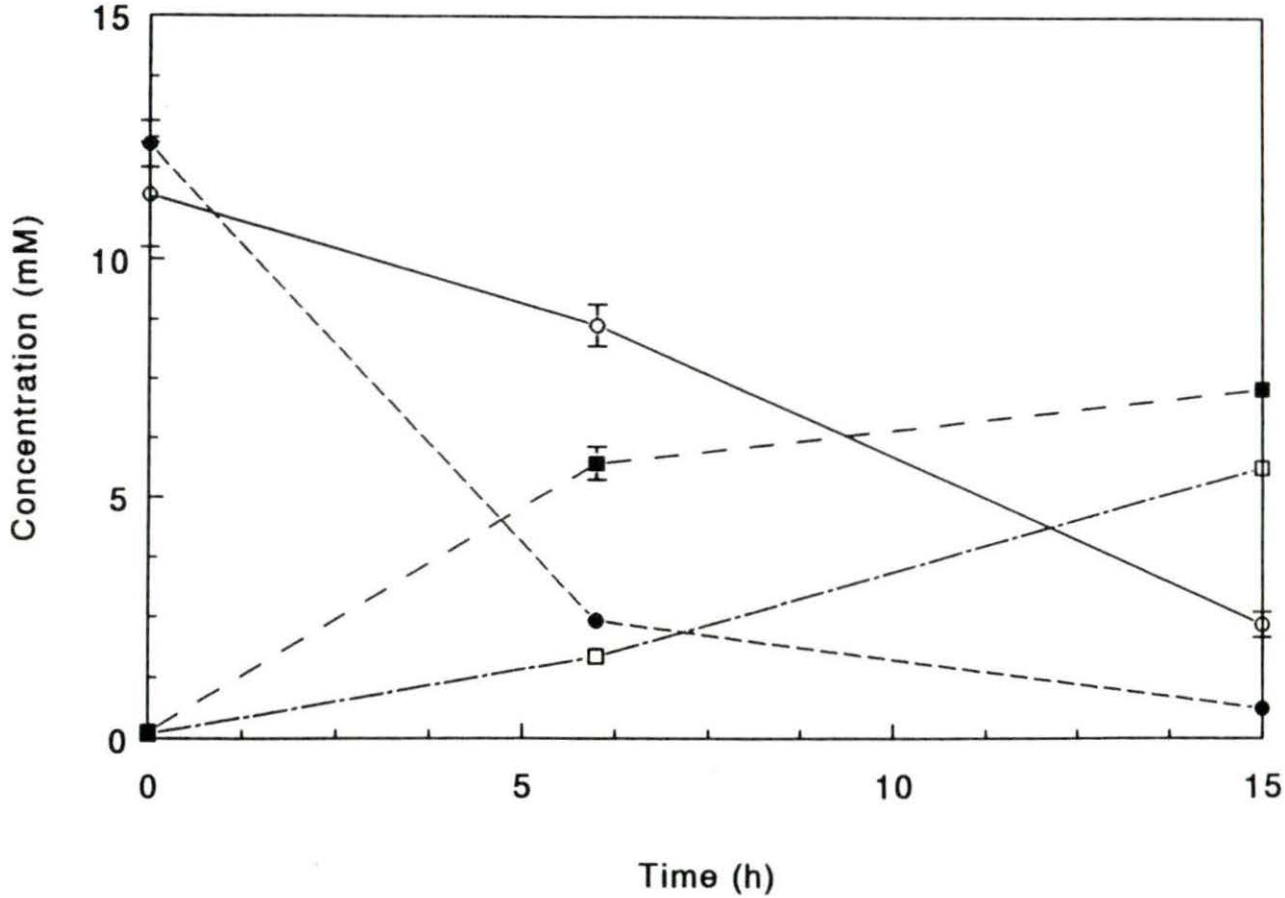
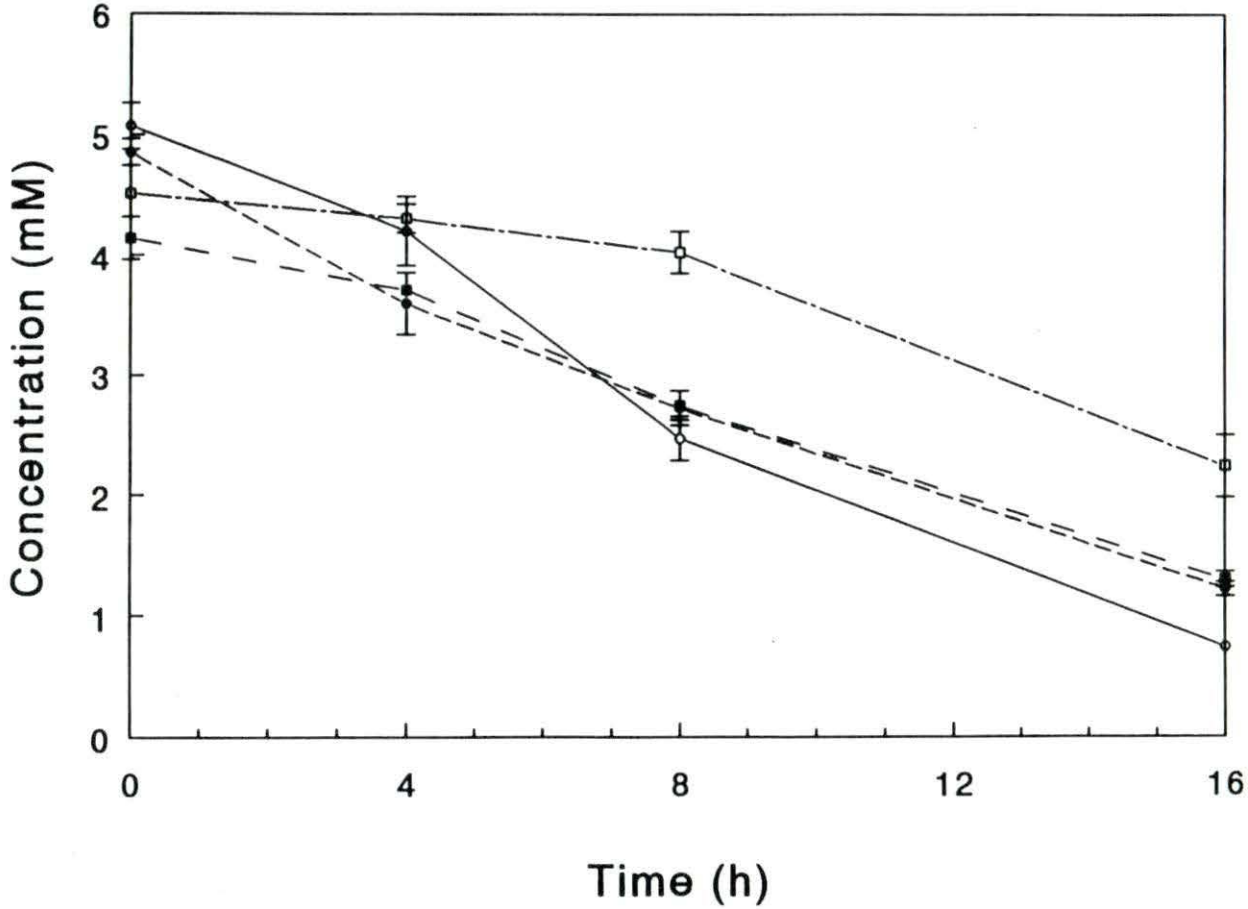

15
10
5
0
Concentration (mM)
0
5
10
15
Time (h)

Figure 5. Changes in nitropropionic acid (circles) or nitropropanol (squares) concentrations during incubation of mixed rumen microorganisms in rumen fluid with (closed legends) or without (open legends) added ferrous sulfate (20 mM) plus sodium sulfide (5 mM) supplement


Concentration (mM)
Time (h)
0
1
2
3
4
5
6
0
4
8
12
16

SUMMARY AND DISCUSSION

The nitro-toxins 3-nitropropionic acid (NPA) and 3-nitropropanol (NPOH) are responsible for poisoning thousands of sheep and cattle each year (49). Ruminal microbes are capable of metabolizing the nitro-toxins; however, the factors affecting this metabolism remain unclear. Our investigations were conducted to elucidate some of the mechanistic processes involved in the microbial metabolism of NPA and NPOH.

The findings presented here demonstrate that the principle metabolic pathway for the microbial metabolism of NPA and NPOH involved an *in situ* reduction, producing β-alanine and 3-amino-1-propanol, respectively. Additionally, β-alanine was, but 3-amino-1-propanol was not, further metabolized by suspensions of ruminal microbes.

A nonspecific hydrogenase/ferredoxin system like that reported by Angermaier and Simon (3) may be functioning in our system because we observed increased rates of NPOH metabolism when ruminal microbes were incubated under a H_2-rich atmosphere and a decrease in NPOH metabolism when ruminal microbes were incubated with carbon monoxide, a known hydrogenase inhibitor.

Small quantities of nitrite may be liberated from the parent compounds during the reduction because we detected

nitrite in our microbial incubations; however, the amounts would account for <1% of the NPA or NPOH metabolized. Nitrite may play an important role by inactivating ferredoxin which was implicated in the reduction. The possible reactivation or stablization of ferredoxin in our system is suggested by the 3-fold increase in NPOH metabolism and 3-amino-1-propanol formation when ferrous sulfate (20 mM) and sodium sulfide (2.5 mM) were added to the microbial incubations. These ions are thought to form biologically active clusters that reconstitute inactivated ferredoxin or apoferredoxin to active ferredoxin.

Although ruminal microorganisms are known to metabolize NPA and NPOH, little is known about the effects of these compounds on the growth of the bacteria. In this regard, we assessed the effects of 4.2, 21, or 42 mM NPA or NPOH on the growth of ruminal bacteria appearing in anaerobic roll tubes. Most of the bacteria grew in the presence of 4.2 mM NPA or NPOH and some could tolerate high levels of the toxins. The lower concentration may be near that expected in the rumen of animals grazing a milkvetch range.

Future research designed to elucidate potential rate-limiting factors involved in the reduction of NPA and NPOH may eventually lead to practical methods of preventing livestock poisonings.

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APPENDIX

Effect of Carbohydrate Addition on NPA Metabolism

Attempts to stimulate the microbial metabolism of NPA by adding a mixture of carbohydrates (glucose, cellobiose, xylose, and soluble starch; 0. 0.1, 0.5 or 1.0% of each) to bovine ruminal microbes in whole rumen fluid were unsuccessful. Our measurements of NPA concentration indicate that addition of the carbohydrate mixtures had little effect on NPA degradation after 3 h but decreased NPA degradation after 8 h (Figure A1). After 20 h incubation at 37^{o}C the pH values were 6.43, 5.90, 4.79 and 4.38 for the cultures incubated with 0, 0.1, 0.5 and 1.0% added carbohydrates, respectively. In order to determine if the decrease in NPA degradation was due to the effect of pH, we incubated mixed bovine ruminal microbes as above except the 1.0% carbohydrate addition was omitted. Additionally, we titrated the pH of the 0.1 and 0.5% added carbohydrate cultures to that of the 0 added carbohydrate culture at each sampling time (except 23 h). A control culture (0.5% added carbohydrate, pH not adjusted) was also tested. The results illustrated in Figure A2 indicate that the addition of the carbohydrate mixtures had little effect on NPA metabolism by ruminal microbes when the pH was adjusted.

Figure A1. Changes in nitropropionic acid concentration during incubation of mixed bovine rumen microorganisms in rumen fluid containing a mixture of added carbohydrates (glucose, cellobiose, xylose, and soluble starch, % of each is indicated within figure); pH was not adjusted during incubation

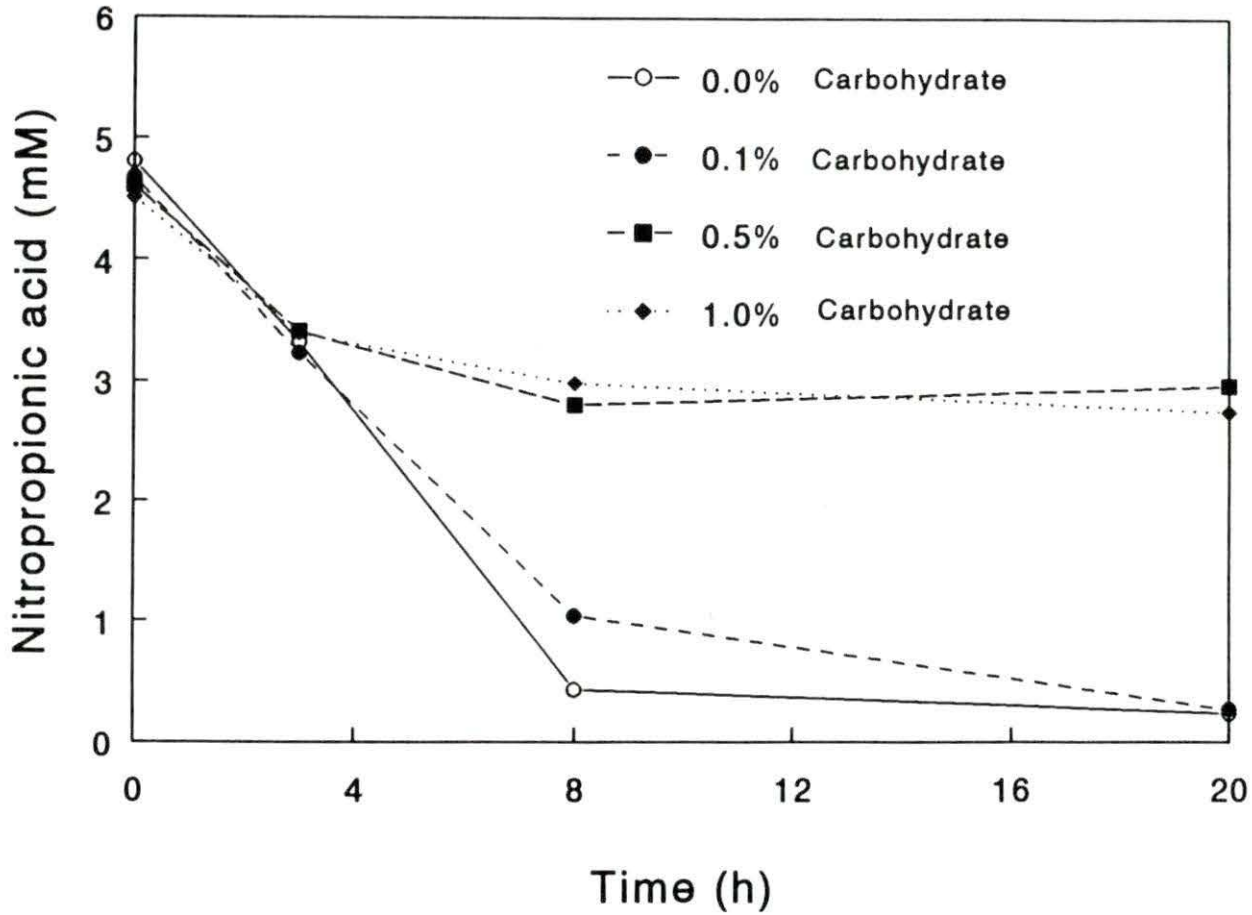
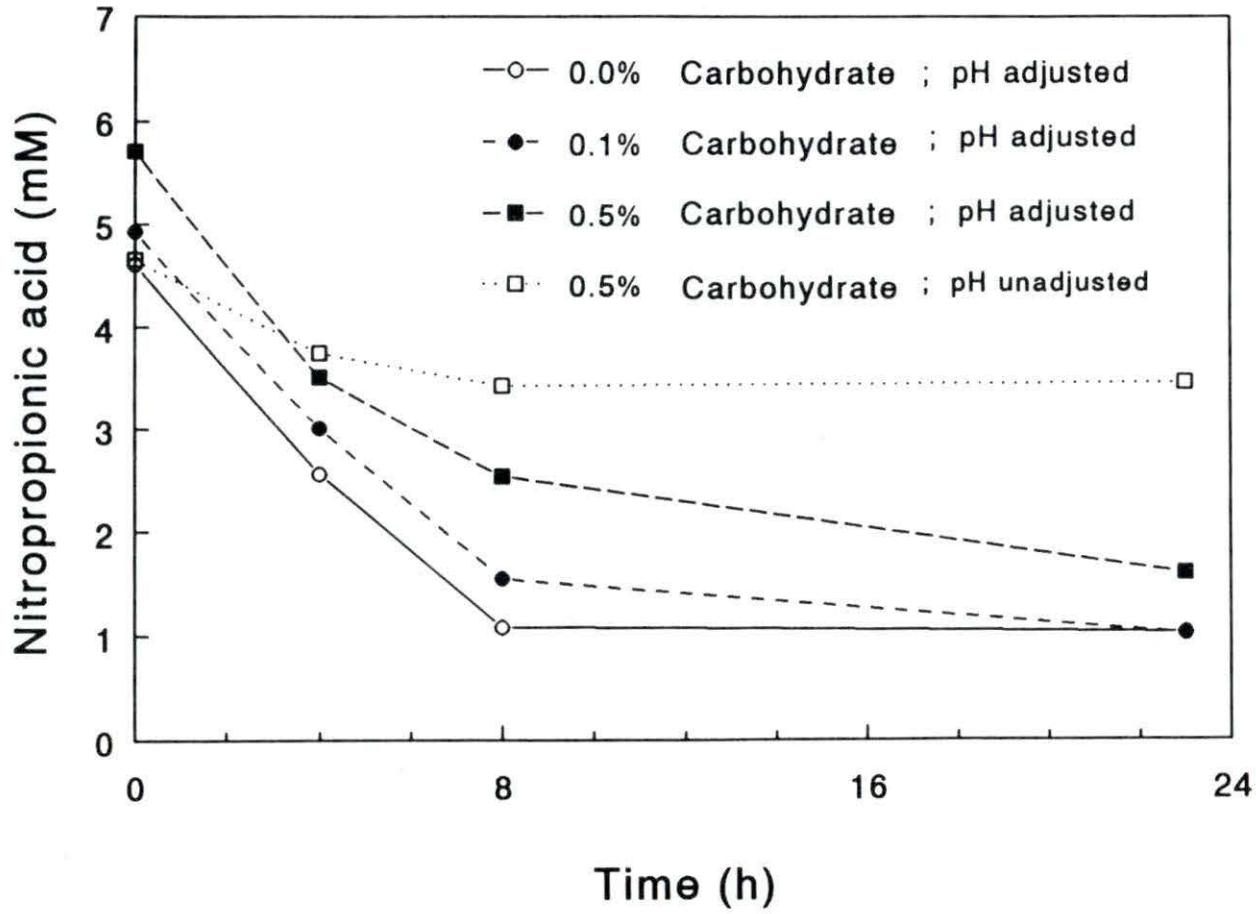

Nitropropionic acid (mM)
6
5
4
3
2
1
0
0
4
8
12
16
20
Time (h)
0.0% Carbohydrate
0.1% Carbohydrate
0.5% Carbohydrate
1.0% Carbohydrate

Figure A2. Changes in nitropropionic acid concentration during incubation of mixed bovine rumen microorganisms in rumen fluid containing a mixture of added carbohydrates (glucose, cellobiose, xylose, and soluble starch, % of each is indicated within figure); pH adjusted at 0, 4 and 8 h to that of 0 addition culture; 0.5% added carbohydrates, pH not adjusted during incubation, is also shown


Nitropropionic acid (mM)
7
6
5
4
3
2
1
0
0
8
16
24
Time (h)
0.0% Carbohydrate ; pH adjusted
0.1% Carbohydrate ; pH adjusted
0.5% Carbohydrate ; pH adjusted
0.5% Carbohydrate ; pH unadjusted

Determination of Protozoal and Bacterial NPA Degradation Activity

While ruminal bacteria have been shown to metabolize nitropropionic acid (NPA) and nitropropanol (NPOH), little is known about the ability of protozoa or fungi to degrade the toxins. From whole rumen fluid, we prepared protozoa rich and bacteria rich suspensions as described below and tested these fractions for their capacity to metabolize NPA.

Two 500 ml separatory funnels, each containing 300 ml of bovine rumen fluid were incubated at 39°C. After 30 min, a white, protozoa rich sediment (approximately 1.5 ml) was collected from each funnel. The fractions were combined and resuspended to 100 ml with anaerobic dilution solution (andil). To reduce bacterial contamination in the protozoal suspension it was centrifuged 5 min at 150 x g and the pellet was resuspended in an equal volume of andil.

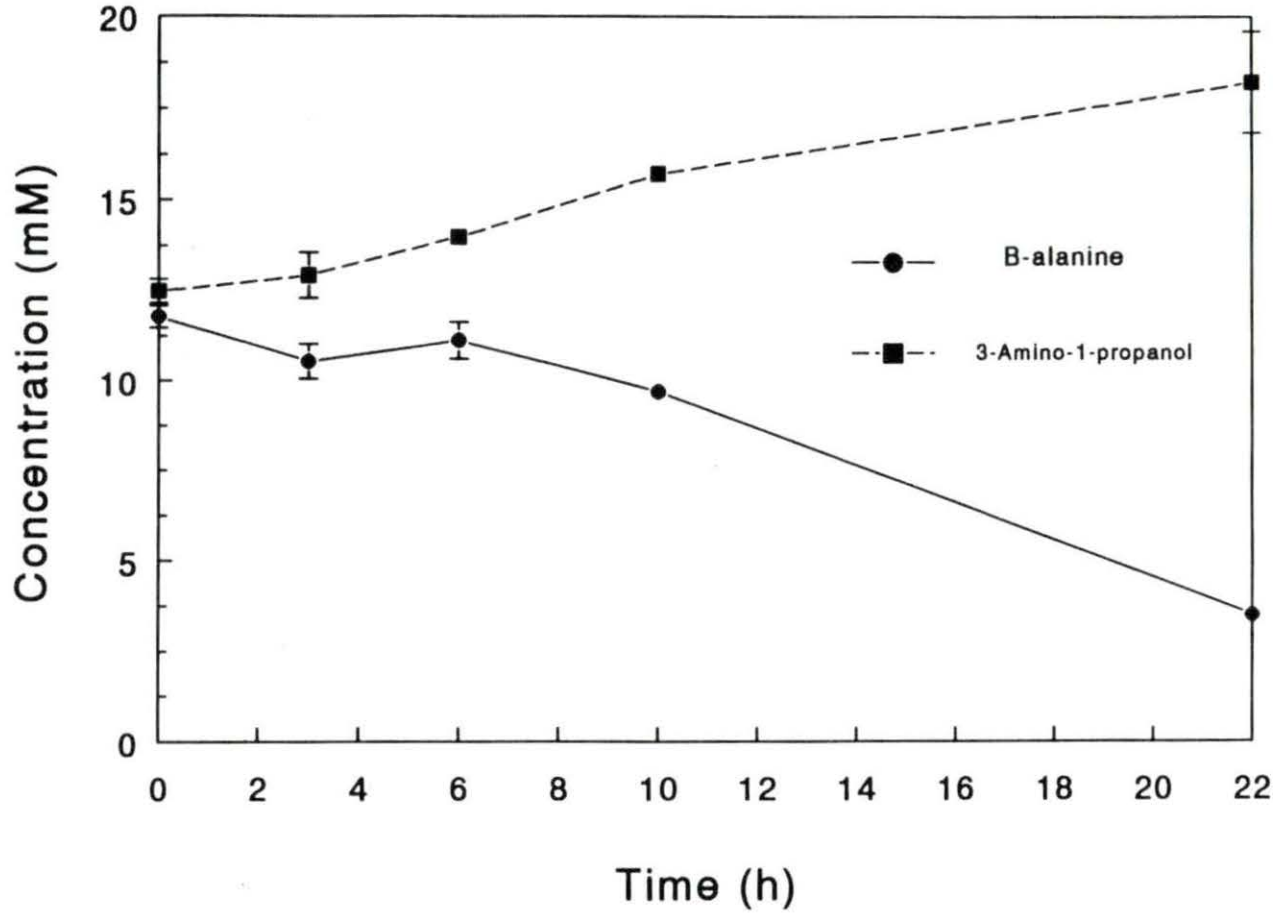
After sedimentation of the protozoa, the rumen fluid remaining in the separatory funnels was centrifuged 10 min at 18,000 x g and the cell population, free from most of the protozoa, was resuspended with andil.

Metabolism of NPA by these protozoa rich and bacteria depleted populations was compared with that by unseparated microbial populations, prepared by centrifuging whole rumen fluid 10 min at 18,000 x g, discarding the supernatant fluid

and resuspending the cells to their initial volume with andil. Aliquots (10 ml) from each of the microbial suspensions were transferred to 18 x 150 mm culture tubes and incubated (37°C) under a 100% CO_2 atmosphere with 4.2 mM NPA. Protein measurements were made from subsamples collected at 0 time and the rates of NPA degradation reported as μmole/mg protein/h.

After 4 h incubation, the bacterial fraction had approximately the same NPA degrading activity (0.110 μmole/mg protein/h ± 0.002) as the unseparated microbial population (0.108 μmole/mg protein/h ± 0.005). No activity was observed in the protozoal fraction, however, the low protein concentration of this fraction (0.48 ± 0.04 mg/ml), as compared the protein concentrations of the bacterial or unseparated microbial populations (3.64 ± 0.01 and 4.52 ± 0.33 mg/ml, respectively), restricts our ability to make conclusions regarding the protozoal NPA metabolic capacity.

Figure A3. Changes in 3-amino-1-propanol and β-alanine concentrations during incubation of concentrated bovine rumen microbial suspensions with 12.6 mM 3-amino-1-propanol or β-alanine


Concentration (mM)
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
22
Time (h)
B-alanine
3-Amino-1-propanol

Determination of Products of NPA and NPOH Metabolism by Ruminal Microbes in Whole Rumen Fluid

We had demonstrated the reduction of nitropropionic acid (NPA) to β-alanine and nitropropanol (NPOH) to 3-amino-1-propanol by 3X concentrated suspensions of ruminal microbes; however, we wanted to demonstrate this process in an environment more closely representing the rumen. Therefore, we incubated ruminal microbes in whole rumen fluid with 4.2 mM NPA or NPOH. We detected 0.81 $\pm$ 0.12 μmole dabsylated β-alanine/ml in subsamples removed after 8 h of incubation with NPA. Dabsylated 3-amino-1-propanol (1.32 $\pm$ 0.03 μmole/ml) was detected in subsamples withdrawn after 16 h incubation with NPOH. These incubation intervals were selected because they were likely to have detectable levels of the reduced products (based on the data obtained from incubations of the 3X concentrated cells) and were the only such samples examined. The presence of β-alanine and 3-amino-1-propanol in these whole rumen fluid incubations with NPA or NPOH were confirmed by TLC (data not shown).

Table A1. Changes in VFA concentration during incubation of mixed rumen microorganisms[a] with or without nitropropionic acid

Hour	Concentration (μmole/ml)[b] Acetate	Propionate	Butyrate	Total
		Without nitropropionic acid		
0	3.8 ± 0.04	0.7 ± 0.01	1.4 ± 0.02	6.0 ± 0.02
3	9.5 ± 0.08	1.9 ± 0.04	4.2 ± 0.01	15.6 ± 0.04
6	12.0 ± 0.16	2.7 ± 0.01	5.7 ± 0.03	20.3 ± 0.18
10	14.9 ± 0.26	3.6 ± 0.02	7.4 ± 0.04	25.9 ± 0.32
22	20.4 ± 0.10	5.2 ± 0.06	9.4 ± 0.23	34.9 ± 0.39
		With nitropropionic acid		
0	3.8 ± 0.11	0.7 ± 0.01	1.4 ± 0.05	5.9 ± 0.17
3	8.7 ± 0.13	1.4 ± 0.01	4.5 ± 0.04	14.6 ± 0.18
6	12.0 ± 0.44	2.1 ± 0.10	6.0 ± 0.20	20.1 ± 0.74
10	14.9 ± 0.33	3.1 ± 0.07	7.2 ± 0.03	25.2 ± 0.43
22	21.1 ± 0.12	6.3 ± 0.06	8.6 ± 0.11	36.0 ± 0.17

[a]Concentrated (3X) mixed bovine microbial suspensions were incubated in anaerobic dilution solution initially containing 0 or 12.6 mM nitropropionic acid.

[b]Mean ± SD from duplicate cultures.

Table A2. Changes in VFA concentration during incubation of mixed rumen microorganisms[a] with or without nitropropanol

Hour	Concentration (μmole/ml)[b] Acetate	Propionate	Butyrate	Total
		Without nitropropanol		
0	3.8 ± 0.04	0.7 ± 0.01	1.4 ± 0.02	6.0 ± 0.02
3	9.5 ± 0.08	1.9 ± 0.04	4.2 ± 0.01	15.6 ± 0.04
6	12.0 ± 0.16	2.7 ± 0.01	5.7 ± 0.03	20.3 ± 0.18
10	14.9 ± 0.26	3.6 ± 0.02	7.4 ± 0.04	25.9 ± 0.32
22	20.4 ± 0.10	5.2 ± 0.06	9.4 ± 0.23	34.9 ± 0.39
		With nitropropanol		
0	3.8 ± 0.02	0.7 ± 0.01	1.1 ± 0.29	5.6 ± 0.29
3	8.3 ± 0.02	2.11 ± 0.01	4.3 ± 0.04	14.7 ± 0.02
6	10.5 ± 0.01	3.1 ± 0.02	6.2 ± 0.01	19.8 ± 0.05
10	12.7 ± 0.09	4.3 ± 0.03	7.8 ± 0.09	24.8 ± 0.22
22	17.9 ± 0.64	6.5 ± 0.21	9.2 ± 0.59	33.6 ± 1.44

[a]Concentrated (3X) mixed bovine microbial suspensions were incubated in anaerobic dilution solution initially containing 0 or 12.6 mM nitropropanol.

[b]Mean ± SD from duplicate.