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Toxicity and Metabolism of the Conjugates of 3-Nitropropanol and 3-Nitropropionic Acid in Forages Poisonous to Livestock

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Glycosides of 3-nitro-1-propanol (nitropropanol) and glucose esters of 3-nitro-1-propanoic acid (nitropropionic acid) occur in many forages distributed throughout the world. Systemically, nitropropionic acid irreversibly inactivates succinate dehydrogenase, thereby blocking ATP formation. Nitropropanol is not toxic per se in mammals but is converted to nitropropionic acid by hepatic alcohol dehydrogenase. Nitrotoxins can be metabolized by rumen microbes, which may provide a mechanism for detoxification. At least 20 different ruminal bacteria are known to metabolize the nitrotoxins, but most appear to play a minor role in detoxification. Evidence suggests that an obligate anaerobic nitro-respiring bacterium, *Denitrobacterium detoxificans*, may be particularly important in conferring protection to animals consuming the nitrotoxins as this bacterium metabolizes the toxins at rates near those by mixed ruminal populations. Rates of ruminal nitrotoxin metabolism can be enhanced by modifying the rumen environment through dietary manipulations, which suggests in vivo enrichment of competent nitrotoxin-metabolizing bacteria such as *D. detoxificans*.

KEYWORDS: Toxic plant; *Astragalus*; *Coronilla*; *Hippocrepis*; *Indigofera*; *Lotus*; toxic fungi; *Arthrinium*; *Aspergillus*; *Penicillium*; 3-nitropropanol; 3-nitropropionic acid; rumen metabolism; *Denitrobacterium*

MAMMALIAN TOXICITY

Nearly a century has passed since Marsh and Clawson (*1*) first reported livestock poisonings caused by plants now known to contain nitrotoxins. Since then, poisonings in cattle, sheep, goats, horses, and insects by plants containing 3-nitro-1-propanol (nitropropanol) or 3-nitro-1-propanoic acid (nitropropionic acid) have been documented (*2*, *3*). Stermitz and colleagues (*4*) isolated and identified the most common glycoside of nitropropanol, miserotoxin, 3-nitro-1-propyl-β-D-glucopyranoside from *Astragalus oblongifolius*, and numerous glucose esters of nitropropionic acid have also been isolated (**Table 1**). Other minor glycosides of nitropropanol have also been isolated, including the β-D-gentiobioside (*14*), allolactoside (*15*), laminaribioside (*16*), and cellobioside (*17*) from *Astragalus miser* var. *serotinus*. *Astragalus canadensis* also contains oxotetrahydrofuranyl and isoxazolinone esters of nitropropionic acid (*18*,

Table 1. Substitution Patterns for Glucose Esters of 3-Nitropropionic Acid (NPA)

common name	position of NPA and anomeric configuration	source	ref[a]
6-monoester	6-α,β	*Coronilla varia*	*5*
cibarian	1,6-β	*Astragalus cibarius*	*6*
coronarian	2,6-α	*C. varia*	*7*
	4,6-α	*Indigofera endecaphylla*	*6*
	4,6-β	*I. endecaphylla*	*6*, *8*
karakin	1,2,6-β	*I. endecaphylla*	*8*
coronillin	1,2,6-α	*C. varia*	*7*
corynocarpin	1,4,6-β	*Corynocarpus laevigatus*	*9*
corollin	2,3,6-α	*C. varia*	*7*
	3,4,6-α	*I. endecaphylla*	*10*
	1,3,6-β	*C. laevigatus*	*11*
hiptagin	1,2,4,6-β	*Hiptage madablota*	*12*
	2,3,4,6-α	*I. suffruticosa*	*13*
	1,2,3,6-β	*C. laevigatus*	*11*
	1,3,4,6-β	*C. laevigatus*	*11*

[a] First proof of structure.

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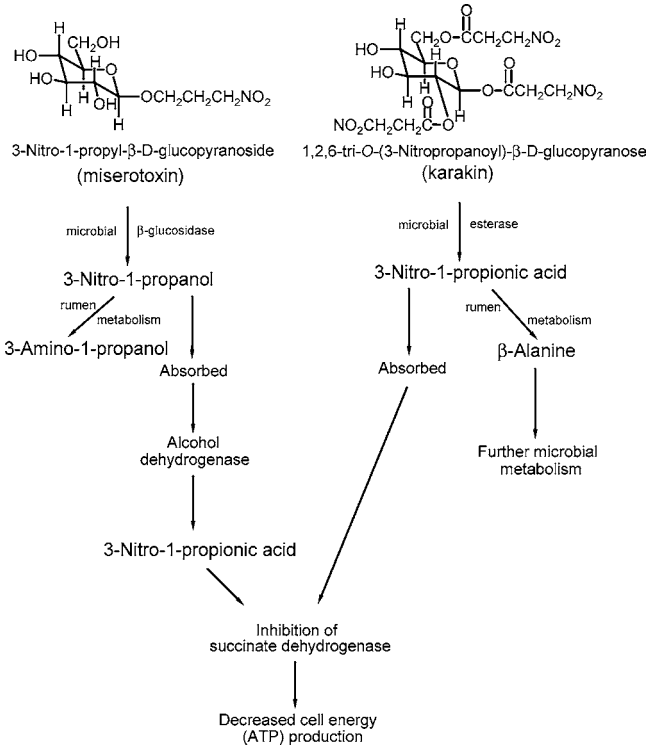

Figure 1. Schematic representation of major events during ruminal and systemic metabolism of nitropropanol and nitropropionic acid in vivo.

19). Specific estimates of the number of livestock poisoned by nitrotoxins are not available, but losses due to all poisonous plants in the western United States exceeded $200 million in 1987 (*20*). The poisoning of humans who consumed moldy sugar cane contaminated with a nitropropionic acid synthesizing fungus, *Arthrinium*, has also been reported (*21*, *22*). The literature is replete on the neurodegenerative lesions induced by nitropropionic acid in the basal ganglia and the pathological linkage to Huntington's disease (*23*, *24*).

In ruminants, the glucose conjugates are rapidly hydrolyzed by microbial β-glucosidase and esterase in the rumen to liberate free nitropropanol or nitropropionic acid (*25*, *26*) (**Figure 1**). Following hydrolysis, nitropropanol and nitropropionic acid are either further metabolized by the ruminal microbes or rapidly absorbed into the circulatory system (*3*). Absorption of the nitrotoxins occurs primarily in the reticulo-rumen (*27*, *28*). In nonruminants, esters of nitropropionic acid are hydrolyzed by mammalian esterase activity in the upper gastrointestinal tract but rapid absorption of the nitroacid precludes its metabolism by microbes in the lower gut (*3*).

In contrast to ruminants, the absence of microbial β-glucosidase activity in the stomach of monogastrics results in intact miserotoxin being absorbed in the upper gastrointestinal tract with little of the glycoside persisting long enough to reach microbial enzyme activity in the lower gastrointestinal tract (*29*). Nonruminant animals are therefore less susceptible to nitrotoxin poisoning by miserotoxin than by free nitropropanol, the aglycone, and this applies to other glycosides as well (*3*). When the toxicities of the aglycone and the glycoside were compared in treated rats, the LD_{50} of miserotoxin was more than 2500 mg/kg body weight as compared to an LD_{50} of 77 mg/kg for nitropropanol (*29*). Rabbits are susceptible to poisoning by miserotoxin, but poisoning was due to methemoglobinemia (*30*) rather than, as described below, by inhibition of succinate dehydrogenase by absorbed nitropropanol (*3*). The methemoglobinemia presumably resulted from the liberation of nitrite within the gastrointestinal tract without hydrolysis of the glycoside. It should be noted that methemoglobinemia was also a secondary disorder in nitrotoxin poisoning in cattle (*31*) and sheep (*32*).

Various animal species have been used to elucidate the toxicity of the nitrocompounds, and poisoning has been experimentally demonstrated in chickens, meadow voles, mice, pigeons, pigs, possums, rabbits, and rats (*3*, *33*). Mechanistically, absorbed nitropropionic acid causes poisoning by irreversibly inactivating succinate dehydrogenase (*34*−*37*) (**Figure 1**). Following absorption, nitropropanol is rapidly and irreversibly converted to nitropropionic acid by hepatic alcohol dehydrogenase (*27*, *38*−*41*). In vitro, nitropropanol could also be converted to acrolein and nitrite (*38*, *39*). The inactivation of succinate dehydrogenase is thought to result from the oxidation of nitropropionic acid to 3-nitroacrylate, which then binds to the enzyme (*35*). Alternatively, Alston et al. (*34*) proposed that the nitronate ion of nitropropionic acid irreversibly binds to the flavin component of succinate dehydrogenase. Fumarase is also inhibited by nitropyropionic acid in vitro (*103*).

There is no antidote for nitrotoxin poisoning. Inhibitors of alcohol dehydrogenase, such as ethanol or 4-methylpyrazole, inhibited the transformation of nitropropanol to nitropropionic acid (*3*), but unless these inhibitors were administered prior to dosing with nitropropanol, poisoning was not prevented. An early report that intramuscular administration of thiamine hydrochloride alleviated signs of nitrotoxin poisoning in cattle (*42*) has been challenged. Administration of thiamine did not diminish the toxicity of nitrotoxins in rats (*43*).

Animals poisoned by the nitrotoxins exhibit a variety of symptoms depending on the severity of poisoning. Acutely poisoned animals may die within 4−25 h (*2*). Acute signs of poisoning include incontinence, loss of hindquarter coordination, and excessive salivation (*44*). Animals poisoned less severely show difficulty in breathing, muscular incoordination, depression, weight loss, and an increased heart rate (*2*, *30*, *31*, *45*−*47*). In chronic poisoning, permanent damage is manifested as unthriftiness, diarrhea, and loss of stamina (*44*). The term "cracker heels" is associated with nitrotoxin poisoning in cattle because the heels often click together when the animals trail (*45*). Frequent urination and frothiness at the nose have also been observed (*48*). Characteristic with poisoned rats and meadow voles is an arching of the back (*49*). Chronically poisoned cattle may remain affected for up to 6 months or permanently and may die at any time if stressed (*45*).

Signs of poisoning of cattle by timber milkvetch (*A. miser* var. *serotinus*) under range conditions in British Columbia were recently documented (*44*). The effects of age and geographic origin of cattle on the incidence of *A. miser* poisoning were also reported. Lactating first and second calf heifers (2 and 3 years old, respectively) were most susceptible. In contrast, older cows or yearling heifers were less susceptible. Cattle originating from the prairie regions of Alberta where *A. miser* does not occur were also more susceptible than cattle from British Columbia where *A. miser* is indigenous (*44*), thus indicating that susceptibility to poisoning by nitropropanol may be reduced by prior exposure to *A. miser*.

DISTRIBUTION OF NITROTOXINS IN PLANTS

In the United States, nitrotoxin poisonings of ruminants occur most often in the Rocky Mountain regions (*45*) and the milkvetchs *A. miser* var. *oblongifolious*, *A. miser* var. *hylophilus*, and *A. miser* var. *serotinus* have most often been implicated. In Canada, nitrotoxin poisoning occurs mainly in the southern

Figure 2. Nitropropanol containing *A. miser* var. *serotinus* (timber milkvetch), which is widely distributed in the southern interior of British Columbia, Canada.

Figure 3. Nitropropionic acid containing *A. canadensis* var. *mortonii*, a Canada milkvetch, which is the most widely distributed species of the genus in North America.

interior of British Columbia. Worldwide, more than 450 species and varieties of *Astragalus* (Leguminosae) (**Figures 2** and **3**) are known to contain either nitropropanol or nitropropionic acid, but not both, with over half of these plants present in North America (*50*−*55*). Other legumes, such as *Coronilla*, *Indigofera*, *Lotus*, and *Hippocrepis*, have also been found to synthesize nitropropionic acid (*3*, *56*−*63*). Nitropropionic acid is also synthesized by certain members of the Malpighiaceae, Corynocarpaceae (**Figure 4**), and Violaceae families as well as by certain species of fungi belonging to *Arthrinium*, *Aspergillus*, and *Penicillium* (*3*, *25*, *64*).

Figure 4. Nitropropionic acid containing *Corynocarpus laevigatus*, karaka tree (**A**) and fruit (**B**), which is found in New Zealand.

Miserotoxin concentrations in *A. miser* var. *serotinus* can vary from 2 to 6% of the plants' dry matter (*65*), and at a minimum lethal dose of 20−60 mg nitropropanol/kg body weight (*66*), a 500 kg cow could be poisoned by eating 3−9 kg of fresh plant matter. It is known that ruminants are more susceptible to poisoning by nitropropanol than nitropropionic acid (*19*). However, some plants that contain nitropropionic acid contain more than twice the amount of toxin found in plants that contain nitropropanol (*45*). Thus, on a dry weight basis, plants containing nitropropanol or nitropropionic acid may be equally toxic.

Several factors affect the levels of nitrotoxins within plants. Plants grown under conditions of restricted sunlight or with suboptimal moisture had reduced miserotoxin levels (*68*). Likewise, Parker and Williams (*69*) demonstrated that plants grown at 24 °C had decreased levels of miserotoxin as compared to plants grown at 32 °C. They also showed that disrupting photosynthesis with (2,4,5-trichlorophenoxy) acetic acid or 2-(2,4,5-trichlorophenoxy) propionic acid resulted in decreased levels of miserotoxin. Miserotoxin levels in *A. miser* var. *serotinus* were unaffected by fertilization with ammonium nitrate, potassium nitrate, or ammonium sulfate, each at 56 and 112 kg N/ha (*69*). In another study, Majak and Wikeem (*65*) found that aerial fertilization with 100 kg urea N/ha did not affect levels of miserotoxin at a grassland site, but at 200 kg N/ha, levels were decreased on a clear-cut site. Miserotoxin levels in plants on the clear-cut increased during the second growing season, but the authors could not rule out the effects of other contributing factors such as reduced interspecific competition, residual nitrogen, or favorable moisture conditions. Concentrations of miserotoxin in established plants are highest prior to flowering and then decrease as the plant matures (*45*, *70*). It should be noted, however, that the overall availability of miserotoxin within a particular range may increase during the growing season because of increases in the biomass of *A. miser* var *serotinus* (*71*, *72*). Consequently, to minimize the risk of poisoning, management strategies should rely on restricting the access of livestock to *A. miser* range during periods of high miserotoxin availability. These studies (*71*, *72*) also showed that

the vetch had poor regrowth characteristics in response to clipping or grazing; consequently, it was suggested that cattle may be less susceptible when grazing a pasture or range that had been previously grazed during the same growing season.

RUMINAL METABOLISM AND POTENTIAL FOR DETOXIFICATION

Most ruminal microbes tolerate relatively high concentrations of the nitrotoxins, with only 10−20% of the population being inhibited by concentrations likely to be present in the rumen of animals grazing a milkvetch range (*73*). Little is known as to the identity of those microbes affected, but evidence now indicates that nitropropanol, nitropropionic acid, and several other less toxic nitrocompounds are potent inhibitors of ruminal methanogenesis (*74*, *75*). When given intraveneously, nitropropanol is lethal to cattle at a dose of 30 mg/kg body weight (*31*) and nitropropionic acid is lethal to sheep at 52 mg/kg body weight (*66*). When administered orally, the lethal doses of nitropropanol in cattle and sheep are nearly doubled, 57 and 118 mg/kg body weight, respectively (*46*, *76*), suggesting the occurrence of microbial metabolism within the rumen. Ruminal microbes are known to metabolize nitropropionic acid more rapidly than nitropropanol (*77*), and as a consequence, the amount of nitropropionic acid required to cause poisoning is 4-fold higher than that of nitropropanol (*32*, *78*). Accordingly, some plants containing the nitroacid can sometimes be safely utilized by ruminants (*25*, *79*−*81*).

Majak and Cheng (*82*) identified several strains of ruminal bacteria capable of metabolizing nitropropanol or nitropropionic acid. Those capable of metabolizing the nitrotoxins included *Bacteriodes ruminicola*, *Desulfovibrio desulfuricans*, *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Veillonella alcalescens*, and species of *Clostridium*, *Coprococcus*, *Lactobacillus*, *Ramibacterium*, and *Peptostreptococcus*. Because all of these organisms also reduced nitrite and because nitrite was found to accumulate in resting cell suspensions of *M. elsdenii* and of mixed ruminal populations, it was suggested that nitrotoxins were cleaved to produce nitrite, which was subsequently reduced to ammonia (*82*). It has since been shown that during detoxification, nitropropanol and nitropropionic acid are reduced primarily to their respective amines, aminopropanol and β-alanine, by mixed populations of ruminal microbes, although nitrite may be formed as a minor product (*73*) (**Figure 1**). Aminopropanol appears to be a final product as it accumulated in the incubations whereas β-alanine was further metabolized by the ruminal microbes (*73*) (**Figure 1**). Earlier, it was documented that a transfer of rumen microbial activity, such as nitropropanol detoxification, can occur between adapted and nonadapted groups of cattle having physical contact or close proximity to each other (*83*).

Grasshoppers exhibit separate yet unique mechanisms for the detoxification of nitropropionic acid and nitropropanol. In melanopline grasshoppers, the glucose ester karakin (**Figure 1**) was hydrolyzed in vivo and after conjugation to glycine, serine, or glutamate, nitropropionic acid was excreted as the amide (*84*). When grasshoppers were fed miserotoxin, the glycoside was primarily eliminated intact (*85*). If nitropropanol was administered, however, the aglycone was detoxified either by glucosylation to miserotoxin or by oxidation to nitropropionic acid with subsequent conjugation to amino acids as above. In both cases, the resultant conjugates were excreted. In contrast, another insect, the domestic honey bee (*Apis mellifera*), is susceptible to nitrotoxin poisoning (*86*).

Rates of ruminal nitropropanol metabolism can be increased by feeding cattle sublethal amounts of *A. miser* or supplements containing nitrate or nitroethane, a less toxic analogue of nitropropanol (*67*, *83*, *87*, *88*). It should be noted that the enhanced metabolism of nitropropanol in the rumen was also reflected in reduced levels of nitropropionate in the circulatory system (*88*). Similarly, rates of nitrotoxin metabolism were markedly enhanced following adaptation of a mixed population of ruminal bacteria to nitropropanol in vitro (*89*). A nitrotoxin metabolizing bacterium capable of metabolizing both nitropropanol and nitropropionic acid, as well as a variety of other nitrocompounds, was isolated from this enriched population (*89*). Upon further characterization of this bacterium, it became apparent that this strictly nonfermentative anaerobe differed markedly from known bacteria in that it obtains energy for growth strictly via anaerobic respiration, oxidizing hydrogen, formate, or lactate for the terminal reduction of the nitrocompounds (*89*, *90*). A new genus and species (*Denitrobacterium detoxificans*) was subsequently proposed to accommodate this bacterium (*91*). In vitro evidence indicates that numbers of this bacterium can be increased more than 10 000-fold during enrichment (*89*), which suggests that bacteria like *D. detoxificans* play a major role in the acquisition of tolerance by ruminants gradually adapted to forages containing the nitrotoxins (*88*, *92*).

Rates of ruminal nitropropanol metabolism can be increased or decreased by feeding cattle different forages even when they do not contain the nitrotoxins, but it is not clear from conflicting results if enhanced rates resulted from the enrichment of nitro-reducing bacteria or due to other less specific mechanisms. For instance, cattle grazing native ranges consisting primarily of Kentucky bluegrass, pinegrass, or bluebunch wheatgrass had microbial populations, which metabolized nitropropanol more rapidly than cattle grazing alfalfa or orchardgrass (*93*). In that study, forage quality was also shown to affect the rates at which microbial populations metabolize nitropropanol, with new orchardgrass pasture supporting higher rates than a 10 year old stand of orchardgrass pasture or cured orchardgrass hay. The crude protein content of the fresh orchardgrass pasture (24% dry matter) was higher than that of the 10 year old stand (16% dry matter), thus suggesting that rates were affected by dietary protein. In support of this, cattle receiving orchardgrass or timothy hay plus 0.5 kg soybean meal/head per day had microbial populations that metabolized nitropropanol 40% faster than cattle not receiving the soybean meal supplement (*88*). The efficacy of feeding protein supplements for the prevention of *A. miser* var *serotinus* poisoning in cattle has since been confirmed in field trials (*44*). The incidence of poisoning was 90% less in the supplemented group than in the control group. The supplement consisted of 32% crude protein in a molasses block.

Rates of nitropropanol metabolism were increased 24% in one experiment when molasses was given as a supplement to cattle grazing orchardgrass pasture (*87*), which suggested that providing a readily available source of reducing equivalents from fermentable substrates might also promote enhanced reduction of the nitrotoxins. Reducing equivalents produced during the fermentation of feedstuffs by mixed ruminal populations are used by *D. detoxificans* to reduce the nitrotoxins (*90*). Lewis (*94*) demonstrated that the fermentation products hydrogen, formate, and lactate, as well several other substrates, were used by mixed ruminal populations to reduce nitrate, and Allison and Reddy (*95*) showed that rapid rates of ruminal nitrate and nitrite reduction require an abundant supply of reducing equivalents generated from fermentable energy sources. Thus, it is possible that the effects of the different diets on nitropropanol metabolism were similarly manifested by providing an abundance of the

needed reducing equivalents. However, rates of nitropropanol metabolism were not increased significantly in other experiments when cattle grazing alfalfa were supplemented with molasses or when cattle receiving alfalfa hay were supplemented with corn or silage (*77*, *93*). Moreover, results from in vitro experiments showed that providing a source of potential reducing equivalents, some which serve as donors for microbial reduction of nitrate, was not enough to stimulate nitropropanol metabolism (*73*). Thus, it is apparent that in some cases ruminal nitrotoxin metabolism may be limited by availability of other necessary substrates or nutrients.

Reduction of nitrate by respiratory (also referred to as dissimilatory) nitrate reductases involves low potential electron carriers. For instance, *D. detoxificans* and numerous nitrate-reducing rumen bacteria such as *Wollinella succinogenes*, *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, and *Veillonella alcalescens* contain cytochromes (*90*, *96*−*98*). Ferredoxins have also been implicated as the electron carrier mediating the reduction of nitrate, nitrite, and the nonspecific reduction of nitroethanol by *Clostridium* (*99*−*101*). In this regard, it has been demonstrated that partially purified preparations of hydrogenase from *Clostridium pasteurianum*, when combined with ferredoxin, reduce nitropropanol and nitropropionic acid (*102*). Reduction of nitropropanol and nitroethanol, but not nitropropionic acid, by the hydrogenase/ferredoxin system was markedly stimulated by ferrous and sulfide ions, which purportedly act by reversing the inactivation of ferredoxin by small quantities of nitrite produced as a side product during the reduction (*99*, *102*). Observations that ferrous and sulfide ions stimulate the reduction of nitropropanol by mixed populations of ruminal microbes (*73*) and by washed cells suspensions of *M. elsdenii* but not *D. detoxificans* (unpublished results) support the concept that a nonspecific hydrogenase/ferredoxin reducing system may operate in the rumen as well. Presently, however, it is not known whether the effect of the different forages on rates of ruminal metabolism may be due to differences in their mineral content or if dietary supplementation with appropriate amounts of ferrous and sulfide ions could be used to prevent poisoning in the field.

Numerous forages distributed throughout the world synthesize glucose conjugates of the respiratory toxins nitropropanol and nitropropionic acid that are toxic to mammals. Ruminants are most susceptible because ruminal microbes hydrolyze the conjugates and rapidly release the nitrotoxins. Ruminal microbes can also reduce and detoxify the nitro alcohol and nitroacid to aminopropanol and β-alanine, respectively. Nitropropionic acid is less toxic to ruminants in part because it is metabolized more rapidly by ruminal microbes. Rates of detoxification can be enhanced with dietary supplements such as protein. Evidence has also shown that rates of ruminal nitrotoxin metabolism can be enhanced via selection of competent nitrotoxin-metabolizing bacteria or via nonspecific mechanisms. A newly identified bacterium, *D. detoxificans*, plays a major role in the anaerobic reduction of aliphatic nitrocompounds. More research is needed to support the development of practical management strategies that could help producers prevent nitrotoxin poisoning in the field.

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