ETIOLOGY OF AMMONIATED HAY TOXICOSIS

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ABSTRACT

Some animals consuming hay treated with anhydrous ammonia have developed neurological signs including hyperexcitability, circling and convulsions. A series of experiments was conducted to identify tentatively the toxin and determine its mode of action. Three out of four sheep fed ammoniated orchardgrass hay (approximately 4% ammonia on a dry basis) developed convulsions. Two of the three sheep died within 18 h of the onset of signs. The concentrations of blood lactate and pyruvate were elevated in the symptomatic sheep (P<.05). A proposed toxin, 4-methyl imidazole, did not induce the syndrome when 750 mg/d (approximately 10 times the dietary amount) were administered orally. Four out of five calves that received milk from cows fed ammoniated oat hay (approximately 5% ammonia on dry basis) displayed hyperexcitability and circling. Concentrations of blood lactate and pyruvate were also elevated in the calves. The crude alkaloid fraction of the toxic milk produced neurological signs similar to those of the calves when injected into mice. A fluorescent compound was found in the alkaloid fraction of toxic milk and ammoniated hay, but not in control milk or untreated hay. The fluorescent compound was quite labile; hence, characterization has been unsuccessful thus far.

(Key Words: Ammoniated Feeds, Hay, Sheep, Calves, Alkaloids, Toxicity.)

Introduction

Treating hay with ammonia has become a widely used practice of many livestock producers. Ammonia is inhibitory to many microbes, thus, it can be used as a preservative; but unlike most preservatives, ammonia actually increases the feeding value of hay (Knapp et al., 1975). Ammonia treatment increases the nitrogen content of forage and increases digestibility of fiber components (Waagepetersen and Vestergaard-Thomsen, 1977; Buettner, 1978). Animals fed ammoniated forage had either higher feed efficiency or higher cost efficiency than did animals fed untreated hay (Saenger et al., 1982; Weiss et al., 1982). No adverse effects of feeding ammoniated forages were reported.

Ammoniation of hay has been adopted rapidly by farmers because of its high economic return; however, problems with feeding ammoniated hay have surfaced recently. Livestock producers in Ohio reported that some animals fed ammoniated hay became extremely nervous and difficult to handle. Afflicted animals would run into fences, gates and other objects or run in circles. Some animals fed ammoniated hay have died. Reports from other states indicate that this is a widespread problem (Simms et al., 1983). All hays that produced toxicity were high-quality grasses such as cereal grain hay, sorghum hay and immature grass hay, treated with relatively large amounts of ammonia (>4% of dry weight). Most research has been conducted on lower-quality hay treated with no more than 3% ammonia.

Clinical signs similar to those reported by farmers were observed when cattle and sheep were fed ammoniated molasses (Tillman et al., 1957; Bartlett and Broster, 1958). A pharmacologically active compound, 4-methyl imidazole, was isolated from ammoniated molasses (Wiggins and Wise, 1955). Signs resembling...
those of cattle fed ammoniated hay and molasses were produced in mice and rabbits when they were injected or fed 4-methylimidazole (Nishie et al., 1970). It has been suggested that methylated imidazoles were the toxic agents in ammoniated hay (Ray et al., 1984; Morgan and Edwards, 1986); however, the toxicity of imidazoles to large animals has not been investigated.

Treating hay with ammonia can increase the human food supply by improving the conversion of forage into high-quality protein. Economics are also strongly in favor of ammoniation. The losses due to the toxicity of ammoniated hay can be great, but the cost of abandoning this technology is also high. Therefore, a series of experiments were conducted to determine the toxic agent, and study its mode of action with the ultimate goal of preventing toxicosis.

Materials and Methods

Sheep Trial 1. Eighteen ewes of different breeds were divided randomly into two equal groups. One group was fed untreated orchardgrass hay harvested at Wooster; the other group was fed orchardgrass hay treated with approximately 4% ammonia on a dry matter (DM) basis. The treated hay was harvested and ammoniated at Jackson Branch Station, Jackson, Ohio. The hay was harvested about 8 mo prior to the start of this experiment. Both hays were harvested as large round bales and were fed in bale feeders. A mixture of trace mineralized salt and dicalcium phosphate (1:1), water and hay were available at all times to the sheep. Treated hay had higher concentrations of nitrogen (3.56 vs 2.88% of DM), and ammonia nitrogen (.91 vs .07% of DM), and a higher pH (8.5 vs 5.7) than did control hay.

Animals were fed the hays for 26 d. Blood samples were taken via jugular venipuncture prior to assignment to treatment and every 3 to 4 d thereafter. Upon showing signs of toxicity, a blood sample was collected. Animals were observed for several hours each day. Blood plasma was analyzed for pyruvic and lactic acids by gas-liquid chromatography (GC) of their methyl esters (Supelco, 1975). Malonic acid was used as the internal standard. Serum was analyzed for sodium, calcium, potassium and magnesium by atomic absorption spectrophotometry. Plasma urea nitrogen was measured using alkaline phenol (Fawcett and Scott, 1960). Serum phosphorus was determined using the molybdovanadate method (AOAC, 1965). Bicarbonate was determined by titration (Henry et al., 1974) and chloride was measured using thiocyanate (Sobel and Fernandez, 1963). All assays were carried out in duplicate except for lactic and pyruvic acids which were single assays.

Data were analyzed statistically with analysis of variance (Harvey, 1960). Sheep were divided in four groups: control, ammoniated hay-normal, ammoniated hay-before signs and ammoniated hay-during signs. If there was a significant F-test for treatment (P<.05), Fishers least significant difference test was used to separate means (Steel and Torrie, 1980).

Sheep Trial 2. After the first experiment, a second trial testing the toxicity of 4-methylimidazole was conducted. Six sheep fed ammoniated hay and six fed control hay were divided randomly into two groups within each diet. Treatments were (three sheep/treatment): untreated hay plus sham dose, untreated hay plus imidazole dose, ammoniated hay plus sham dose and ammoniated hay plus imidazole dose. Sheep were orally dosed with 750 mg/d of 4-methylimidazole or corn starch for five consecutive days via gelatin capsules. Sheep were observed for a total of 10 d.

Hay was sampled every 3 or 4 d and composited. Hay was extracted for 4-methylimidazole by the method of Wilks et al. (1977), except that the GC column was packed with 10% Carbowax 20M/2% KOH on Chromosorb W AW instead of 7.5% Carbowax 20M/2% KOH used by Wilks et al. (1977). The internal standard was 2-methylimidazole. Recovery of 4-methylimidazole added to control hay was 97%.

Milk Trial. Two low-producing Holstein cows were fed oat hay treated with approximately 5% ammonia on a DM basis plus trace mineralized salt and dicalcium phosphate ad libitum. The hay was provided by a local farmer and was treated about 18 mo prior to the start of this experiment. After 5 d on trial, milk (designated treated milk) was collected and frozen after each milking. Newborn bull calves were assigned to control milk (five calves) or treated milk (five calves). Calves were fed milk at 10% of body weight/day divided into two equal feedings. No other feed was available to the calves. Calves remained on trial for 15 d. Samples of venous blood were collected from calves at 5-d intervals. Whole blood was assayed...
in duplicate for lactic and pyruvic acids using the enzymatic procedure of Sigma (1984).

Milk and hay were analyzed for alkaloids using a modification of a procedure of Twitchett et al. (1978). Enough 1 N NaOH was added to 75 ml of milk (or 10 g hay plus 50 ml water) to raise the pH to 10. The sample was then extracted twice with 100 ml n-heptane containing 2% isoamyl alcohol. The solvent phase was saved and evaporated to approximately 5 ml (under nitrogen at 35°C). The organic phase was then back-extracted with 0.2 ml of 0.1 N HCl. All extractions were carried out in a dimly lit room. The acid phase was injected (100 μl) via a loop injector into a high-performance liquid chromatograph (HPLC). The HPLC was equipped with a reverse phase column (5 μm, 4.6 × 250 mm, Regis Chem. Co., Morton Grove, IL) and a fluorescence detector (excitation wavelength, 330 nm; emission wavelength, 525 nm). Solvent was 45% methanol and 55% 0.025 M phosphate, pH 7.0, pumped at 1 ml/min.

Biological activity of the alkaloid extracts was tested by subcutaneously injecting 13-g male Swiss mice with extracts of control or toxic milk. The milks were extracted as described previously except that the volumes of milk and organic solvent were doubled. The acid phase of the extract (about 0.2 ml) was injected into the mice.

Results

Sheep Trial 1. All sheep appeared normal from d 0 to 12 of the trial. Signs of toxicity developed on d 13 in three of the nine ewes fed ammoniated hay (33%), two of which died (22%). First, afflicted sheep would stand very still, then their faces would start twitching. The twitching progressed to general body tremors. Opisthotonus (rigid stance with retraction of the head) developed gradually and then, in severe cases, the sheep would go down and convulse violently with grinding teeth, frothing at the mouth and paddling of the legs. Following convulsions or opisthotonus, the sheep would walk in circles with a very rigid gait. Each episode, from twitching to convulsions, lasted less than 1 min. Convulsive episodes recurred every 10 to 15 min. As the toxicity progressed, convulsion recurred more frequently.

An afflicted sheep that was in a comatose condition was killed by electrocution 7 h after onset of signs. A second sheep was found dead at 18 h after onset of signs. Necropsy revealed no gross lesions except for a mildly fatty liver in one sheep. Sections of brain, liver, kidney, lung, heart, muscle and the digestive tract were examined, but no microscopic lesions were found.

One sheep that showed signs of toxicity survived. That sheep did not have as many convulsive episodes as the other two sheep and the convulsions were not as severe. The surviving sheep occasionally ran or walked in circles during the 3 d following the onset of signs. The sheep appeared normal from d 18 and its blood values were similar to control sheep on d 18. No other sheep showed any signs of toxicity during the remaining 13 d that sheep were fed ammoniated hay. Sheep consumed hay from the same large round bale for a total of 26 d, but the three sheep affected by toxicity developed signs within a 1-h period on d 13. The time distribution for the development of signs suggests that the toxin was not distributed uniformly throughout the bale. The reason that only three sheep developed signs could be related to animal differences in dry matter intake and susceptibility to the toxin. The sheep were group fed, therefore individual intake could not be measured. Average DM intake (not corrected for waste) was 1.6 kg/d.

Serum calcium, potassium, phosphorus and chloride were within generally accepted ranges (Smith et al., 1978) during the entire trial and were not affected by toxicity. Plasma urea nitrogen was higher in the ammoniated hay group (20 vs 14 mg/dl) than in the control group, but was not excessively high. Serum sodium, magnesium, bicarbonate, lactic acid and pyruvic acid of all sheep during the first 12 d of the trial were similar to pre-treatment values and were within acceptable ranges, but when toxicity developed those variables were affected (table 1).

Sheep Trial 2. No peak at the retention time of 4-methyl imidazole (7.3 min) was found in control hay. Ammoniated hay contained 53 ± 6 ppm (DM basis, mean ± SE of four assays of the same composite sample) 4-methyl imidazole assuming the peak at 7.3 min was 4-methyl imidazole. Sheep fed ammoniated hay consumed an average of 85 mg of 4-methyl imidazole
<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Nonsymptomatic</th>
<th>Symptomatic Before signs</th>
<th>Symptomatic During signs</th>
<th>SE</th>
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<td>6</td>
<td>3</td>
<td>3</td>
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<td>No. of observations(^a)</td>
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<td>30</td>
<td>12</td>
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<td>Ca, mg/dl</td>
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<td>9.7</td>
<td>9.9</td>
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<td>Mg, mg/dl</td>
<td>2.2(^d)</td>
<td>2.4(^d)</td>
<td>2.5(^d)</td>
<td>3.0(^e)</td>
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<td>K, meq/liter</td>
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<td>5.0</td>
<td>5.2</td>
<td>.5</td>
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<tr>
<td>Na, meq/liter</td>
<td>147.0(^d)</td>
<td>148.0(^d)</td>
<td>146.9(^d)</td>
<td>159.0(^e)</td>
<td>1.9</td>
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<tr>
<td>Total cations(^b), meq/liter</td>
<td>159.0(^d)</td>
<td>159.7(^d)</td>
<td>158.2(^d)</td>
<td>171.7(^e)</td>
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<td>Chloride, meq/liter</td>
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<td>102.0</td>
<td>101.0</td>
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<td>Bicarbonate, meq/liter</td>
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<td>31.2(^d)</td>
<td>30.8(^d)</td>
<td>23.9(^e)</td>
<td>.7</td>
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<td>Anion gap(^c), meq/liter</td>
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<td>25.9(^d)</td>
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<td>144(^f)</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>2.9</td>
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\(^a\)Experimental unit.

\(^b\)Total cations include Ca, Mg, Na and K. Anions include bicarbonate and chloride.

\(^c\)Anion gap = (meq/liter Na + meq/liter K) - (meq/liter bicarbonate + meq/liter Cl).

\(^d,e,f\)Means in same row with unlike superscripts differ (P<.05).
based on an average intake of 1.6 kg hay DM/d, and assuming that samples of hay assayed for 4-methyl imidazole were representative. Sheep were fed 750 mg/d of 4-methyl imidazole (20 mg/kg body weight), which was much lower than the oral LD50 for mice (370 mg/kg, Nishie et al., 1969), but was about 10 times higher than the amount sheep should have received from their diet. Neither sheep fed untreated hay plus imidazole, nor sheep fed ammoniated hay plus imidazole, showed signs of toxicity during the 10-d trial. Based on this trial, it is unlikely that 4-methyl imidazole was the toxic agent in ammoniated hay.

Milk Trial. The cows consumed the hay readily (approximately 16 kg DM·head⁻¹·d⁻¹) and showed no adverse effects. Hay intake and milk production tended to decrease over the 40-d feeding period (due to stage of lactation). No control calves showed any unusual behavior, but four of five calves (80%) fed treated milk displayed severe neurological aberrations. One calf fed treated milk died 3 d after first displaying signs. Calves had no access to ammoniated hay; therefore, the toxin must have been in the milk, a potential risk to human health. Afflicted calves ran in circles and into the walls of their pens, and were very sensitive to noise and touch. A loud noise (e.g., slamming a door) usually precipitated an episode of circling. Signs developed usually within 1 wk of first receiving the treated milk, but one calf did not show any signs of toxicity until d 11. Extreme excitability lasted 2 or 3 d and then calves appeared normal even though feeding of treated milk continued. This suggests that either calves adapted to the toxin, or the toxin was not distributed uniformly in the milk consumed by the calves or in the hay consumed by the cows. Blood lactic and pyruvic acids were elevated in the afflicted calves, but only for about 18 h after signs first developed (table 2).

Analysis of milk for alkaloids with HPLC revealed that a fluorescent compound was present in treated milk but not in control milk (figure 1a, b). The compound of interest eluted at 8.1 min with the HPLC system described previously. An 8.1-min peak also was found in extracts of ammoniated hay, but the hay contained many fluorescent compounds. Milk collected from cows 1 d after their diets were changed from ammoniated hay to untreated hay had only a small peak at 8.1 min. Milk collected 1 wk after the diet change contained no 8.1-min peak.

<table>
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<th>Item</th>
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<th>Case of signs</th>
<th>Onset of signs</th>
<th>Postonset</th>
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</thead>
<tbody>
<tr>
<td>No. of calves</td>
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<td>4</td>
<td>5</td>
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<tr>
<td>Pyruvic acid, mg/d</td>
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<td>17.4b</td>
<td>14.9b</td>
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<td>Lactic acid, mg/d</td>
<td>34.40c</td>
<td>34.40c</td>
<td>16.50b</td>
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</tbody>
</table>

*Experimental unit. Means within row with unlike superscripts differ (P<0.05).
loids as described previously and injected into the HPLC. The 7- to 10-min fraction of the column eluent was collected (4 ml total volume) and then concentrated by evaporation (20°C, under nitrogen in a dark room) to a final volume of about .2 ml. Purity of the evaporated fractions was then tested by injecting the concentrated fraction back into the HPLC. This analysis revealed that the 8.1-min peak had largely disappeared, and a peak at 12.2 min was now evident (figure 1c). Certain alkaloids are quite labile and the production of artifacts during extraction and separation are not uncommon (Twitchett et al., 1978). No response was noted when the 7- to 10-min fraction was evaporated and injected into mice.

Discussion

Sheep fed ammoniated hay and calves fed milk produced by cows fed ammoniated hay developed neurological disorders. The clinical signs were different between species, but blood parameters were similar. The electrolyte data from the first sheep trial was indicative of an acid-based imbalance. The anion gap (sodium plus potassium minus bicarbonate plus chloride) is usually about 20 meq/liter, and becomes larger during metabolic acidosis and smaller during metabolic alkalosis (Ott and Carroll, 1977). The anion gap was about three times larger in symptomatic sheep than in unaffected sheep. The direct cause of the acidosis was the higher concentration of organic acids in the blood. Animals compensate for metabolic acidosis by increasing their respiration rate so that carbon dioxide is removed from the blood. This shifts the equilibrium so that production of water and carbon dioxide from carbonic acid is favored, thereby removing protons from the blood (Guyton, 1976). Respiration rate of the sheep was not measured, but it was very rapid in sheep during convulsive seizures. This would explain the decreased serum bicarbonate of symptomatic sheep.

The increased serum sodium of symptomatic sheep could be due to another compensatory mechanism. In severe states of acidosis, protons are removed from the blood by the excretion of ammonium (derived from glutamine). As ammonium is excreted, sodium is reabsorbed by the kidney to maintain charge balance in the urine (McGilvery, 1979). This could cause serum sodium to increase in acidotic animals. Packed cell volume averaged 38% for all sheep.
and was not affected by toxicity; thus, the changes in serum constituents were not due to alterations in hydration state of the animals.

The marked elevation of pyruvic and lactic acids found in the first sheep trial and the calf trial appeared to be the key to the acid-base imbalance. At least three different hypotheses could explain the increase in blood acids: 1) anaerobic metabolism during severe muscular activity, 2) interference with thiamine metabolism or 3) interference with carbohydrate metabolism caused by the presence of a toxic alkaloid.

Severe muscular activity causes an increase in the concentration of lactic acid in blood (Oliva, 1970); afflicted sheep and calves were extremely active. It is unlikely, however, that muscular activity was the major cause of the increased concentration of blood lactate because blood lactate concentrations in afflicted calves returned to pre-symptomatic values within 18 h of the onset signs, even though calves still displayed hyperexcitability. Also in cases of tissue hypoxia, as found during severe muscular activity, the lactate:pyruvate ratio increases (Huckabee, 1958). In both the sheep and calf trials, lactate and pyruvate concentrations increased and the ratio remained fairly constant.

The second hypothesis that would explain both the clinical signs and the high concentrations of pyruvate and lactate is that thiamine metabolism was inhibited. Consumption of thiamine-deficient diets and some thiamine analogs can produce opisthotonus, convulsions and high blood concentrations of organic acids (Benevenga et al., 1966; Loew and Dunlop, 1972). Attempts to prevent or ameliorate the toxicity with oral or parenteral administration of thiamine failed (Weiss, 1985).

The final hypothesis examined was that a toxic alkaloid, possibly an indole, was the causative agent. Certain indole alkaloids can produce clinical signs in animals similar to those produced by ammoniated hay toxicity. Ergot molds (Dillon, 1955), penicillium tremogens (Cole et al., 1972) and some indolealkyamines (Gallagher et al., 1966) cause hyperexcitability, circling and convulsions in animals. Furthermore, some indole alkaloids cause the concentrations of lactate and pyruvate in the blood to increase (Cysewski et al., 1975). Data from the present experiments are interpreted to support the toxic alkaloid hypothesis. Pyruvate and lactate concentrations were elevated in afflicted animals, and the alkaloid fraction of milk produced by cows fed ammoniated hay was toxic to mice and produced clinical signs identical to those of calves fed the whole milk.

Identification of the alkaloid causing the toxicity has been unsuccessful thus far. With the chromatographic and detection system used, the only compound unique to toxic milk as compared to normal milk corresponded to the 8.1-min peak. The alkaloid fraction of milk produced 1 d after cows were switched from ammoniated hay to untreated hay did not contain an appreciable amount of the 8.1-min compound, and the fraction was not toxic to mice. The 7- to 10-min fraction of the alkaloid fraction following concentration via evaporation also did not contain an appreciable quantity of the 8.1-min peak, and also was not toxic to mice. The toxin was in the alkaloid fraction, and circumstantial evidence implicates the 8.1-min peak as the toxic agent.

The lability of the 8.1-min compound makes it difficult to study. The toxin was not 4-methyl imidazole and, based on retention times (in parentheses) on the HPLC system, the 8.1-min or 12.2-min peaks were not lysergic acid (3.4), 4-methyl imidazole (5.0), bufotenine (6.1) or tryptamine (no peak with fluorescence detection). The milk and hay was assayed for lysergic diethyl amide with thin-layer chromatography by a commercial lab; results were negative. Extraction and separation procedures will have to be modified so that the integrity of the compound eluting at 8.1 min can be maintained and characterization studies can be conducted.

Literature Cited


