EFFECT OF MOLYBDENUM AND COPPER IN FORAGE ON NITRATE REDUCTION IN RUMINANTS

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CHAPTER I

INTRODUCTION

Nitrate poisoning of livestock caused by an excessive intake of nitrate from plants or water has been recognized for over 70 years. The symptoms of nitrate poisoning in livestock include rapid, shallow breathing, wobbly gait, decreased milk production, abortion and death. Nitrate is the major nutrient form in which nitrogen is absorbed by plants and in the great majority of cases, it is assimilated so rapidly that its concentration rarely rises above 0.03%. When conditions occur which retard growth of the plant, the nitrate content may rise to 5% or higher on the dry matter basis. Water containing nitrate has also been responsible for poisoning cattle and sheep.

The native muck soils of Florida contain high levels of molybdenum and nitrate but very low levels of copper. Molybdenum toxicity of cattle grazing forages grown on these soils has been recognized and its alleviation by supplementation of cattle with copper has been established. Feed-lot trials with young dairy bull calves fed levels of molybdenum as high as 100 and 200 ppm mixed in their rations for 22 weeks had no ill effects. This indicated that molybdenum toxicity was more complex than was generally recognized. Nitrate reductase contains molybdenum, and copper has a similar relation to nitrite reductase. With high concentrations of nitrate in the forages grown on the muck soils of Florida, the rate of nitrate reduction to nitrite in the rumen may be related to

the concentration of molybdenum present. The nitrite may accumulate due to lack of copper and thereby increase the hazard of a moderate concentration of nitrate. The nitrite, upon absorption into the blood, may have contributed to the molybdenosis symptoms reported in cattle on these muck soils by oxidizing the iron of hemoglobin to the ferric state forming methemoglobin.

The purpose of the present study was to determine if different levels of molybdenum and copper were practical factors in the rate at which nitrate in forage is reduced in the rumen of cattle and sheep.

Cattle and sheep were fed forages especially grown to contain high nitrate and varying levels of molybdenum and copper. The reduction of nitrate with varying concentrations of molybdenum and copper was also studied *in vitro*.

CHAPTER II

LITERATURE REVIEW

The Toxic Effects of Nitrate on Animals

The first authentic and detailed account of nitrate toxicity of livestock was that of Mayo (1895). Corn (Zea mays L.) was grown in an abandoned hog yard in the dry year of 1895. This corn fodder was fed to cattle late one afternoon and about six hours later some of the cattle were dead while others had collapsed. Potassium nitrate salt was isolated from the corn stalks in amounts as high as 18.8% of the dry matter. He called this toxicity "potash poisoning" and in so doing inadvertently placed a stigma on the cation component which has remained to the present time (Allaway, 1963).

Bradley et al. (1940) showed that the toxic factor of "potash poisoning" was the nitrate ion as distinguished from its potassium or sodium salt. They extracted poisonous oat hay with water and it produced typical nitrate intoxication symptoms when administered to calves. They also found methemoglobin in the blood of these calves.

The following is a list of symptoms which have been caused by excessive dietary nitrate.

Acute Toxicity

Initial symptoms

1. Unsteadiness, wobbly gait and muscle tremor.

2. Severe anoxia.

3. Severe cyanosis marked by rapid, shallow breathing and weak, rapid pulse.

4. Cows apparently blind.

5. Mania.

6. Methemoglobin concentration increased in blood.

7. Dehydration and diuresis with high concentration of nitrate in the urine.

8. Frequent drinking.

9. Gray-brown discoloration of white skin and mucous membranes.

Terminal symptoms

1. Motor spasms (convulsions) progressing to death.

2. Abortion of fetus if present.

3. Death.

Chronic Toxicity

1. Decreased milk production.

2. Rough hair coat.

3. Poor growth and appetite.

4. Lowered conception rate.

5. Small offspring.

6. Irritable.

7. Increased heart and respiration rate.

8. Moderate levels of methemoglobin.

9. Vitamin A deficiency symptoms.

10. Degeneration and fatty infiltration of liver, degeneration of vascular tissues of brain, heart, lungs, kidneys, and testes.

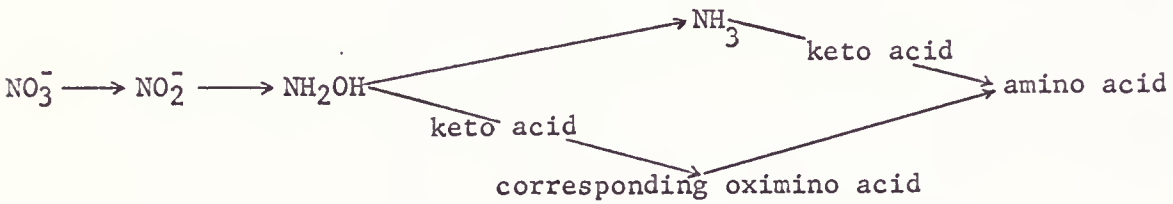
Nitrate Accumulation by Plants

The chemically combined nitrogen absorbed by plants is almost entirely in the nitrate form. Nitrate accumulation by plants thus implies that the rate of utilization has not kept pace with the rate of absorption. The accumulation is often only temporary, due to dry weather, cloudy skies, or any condition that slows photosynthesis. The nitrogen of the nitrate ion undergoes an 8-electron change of valence, from +5 to -3 as shown in the following outline by Nason (1962):

$$NO_3^- \longrightarrow NO_2^- \longrightarrow (HNO),\ NO_2 \cdot NH_2 \text{ or } H_2N_2O_2 \longrightarrow NH_2OH \longrightarrow NH_3$$

$$+5 \quad +3 \qquad\qquad +1 \qquad\qquad -1 \quad -3$$

The nitrate is presumably built into amino acids by one of the two methods described by Wood (1953):



In 1935 Eggleton studied the absorption of inorganic nitrogenous salts by grasses. When plants, which had been growing on unmanured soil, were given a solution of $NaNO_2$, $NaNO_3$, $(NH_4)_2SO_4$ and K_2SO_4, nitrite ion could be detected in the plants two hours later. He stated that this accumulation of nitrite was undoubtedly conditioned by rapid absorption of nutrients and low intensity of solar radiation similar to the situation prevailing in spring, fall and under heavy shade in summer. Olson and Moxon (1942) found that if oat (Avena sativa L.) hay and red root (Amaranthus sp.) hay, which contained high levels of nitrate, were moistened with water and held at room temperature for several days, high

levels of nitrite would be formed. Bacillus subtilis was isolated from the red root hay and was found to be very effective in reducing nitrate to nitrite. These are two of the very few reports in the literature where nitrite was found in forage. Wright and Davison (1964) reported that nitrite was found in very few plants containing high nitrate. Smith (1963) completed numerous studies with corn that was fertilized with high levels of nitrogen and although the nitrate ion content was as high as 4.8% in some samples, only negligible amounts of nitrite were found in any of the forages he examined.

In 1958 Kretschmer found up to 4.7% nitrate ion in oats which were over-seeded in St. Augustine grass (Stenotaphrum secundatum (Walt.) Kuntze) which had been sprigged in the summer on virgin peaty-muck at the Everglades Experiment Station in Florida. Griffith (1958, 1960), Crawford and Kennedy (1960), Perez and Story (1960), Cullison et al. (1962) and Breniman (1963) have reported that high levels of nitrate in forages were caused by high rates of nitrogen fertilization.

Another factor which caused nitrate accumulation in forages was excess shading by high mountains (Gilbert et al., 1946) and by high plant populations (Jordan et al., 1963; Gordon et al., 1962). Shaded forages were relatively low in carbohydrate content due to a decreased rate of photosynthesis. Gordon et al. (1962) explained that utilization of nitrate within plants apparently depended on reduced nicotinamide adenine dinucleotide (NADH) derived from carbohydrate respiration. Therefore, any factor which decreased the production of NADH would enhance the accumulation of nitrate. The converse of the above situation was studied by Hamner (1936). Hamner (1936) washed the roots of 6-in. tomato (Lycopersicon esculentum Mill.) plants and put them in sand containing

no nitrate. The starch content of the leaves rose to a "high level" and there was little or no nitrate present. Nitrate added to the soil was present at the top of the plant six hours later. Following nitrate application the respiration rate was appreciably increased. The treated plants were greener and larger 14 hours after the addition of nitrate.

The application of herbicides has been found to increase the nitrate content of certain forages. Swanson and Shaw (1954) observed a slight increase in nitrate level of young growing sudan grass (Sorghum vulgare var. sudanense (Piper) Hitchc.) when 2,4-D was applied. Berg and McElroy (1953) applied 2,4-D to oats, bromegrass (Bromus inermis Leyss.), timothy (Phleum pratense L.), alfalfa (Medicago sativa L.), red clover (Trifolium pratense L.), sweet clover (Melilotus alba Med.) and white Dutch clover (Trifolium repens L.) and found no significant increase in nitrate levels. However, they did find high nitrate content in Canadian thistle (Cirsium arvense (L.) Scop.), dandelion (Taraxacum officinale (L.) Weber), lambs quarter (Chenopodium album L.), red root pigweed, Russian pigweed (Axyris amaranthoides L.) and Russian thistle Salsula kali L.) when 2,4-D was applied. Frank and Grigsby (1957) also found excess nitrate accumulation due to herbicides in many of the weeds that they studied.

Sund and Wright (1957) and Simon et al. (1959) found increased numbers of abortions when cattle grazed excessively weedy pastures. They found that red-berried elder (Sambucus pubens Michs.), goldenrod (Solidago sp.), stinging nettle (Urtica sp.), boneset (Eupaturium perfoliatum L.), red root pigweed, lambs quarter, burdock (Arctium minus Schk.), Canadian thistle and bull thistle (Cirsium vulgare (Savi) Airy-Shaw) accumulated much higher levels of nitrate than plants of other

species growing in the same field. When these weeds were eliminated from a low-land pasture which had caused abortions for many years, no more abortions occurred.

There may be a varietal difference in nitrate accumulation by forages (Gul and Kolp, 1960). They planted several varities of oats at Laramie and Archer, Wyoming. Variety, as well as location and stage of growth, had a significant effect on nitrate concentration. They stated that it may be possible to select and breed oat varieties which would have lower levels of nitrate. This problem was discussed at the Conference on Nitrate Accumulation and Toxicity in New York City, April 15-16, 1963. At this meeting Dr. Griffith stated that he thought selection and breeding should not be made on the basis of resistance to accumulation of nitrate but rather on the potentiality for production of water-soluble carbohydrate since the latter is inversely related to nitrate content and is an inherited character. Hageman's (1963) work with corn hybrids, which contained high and low levels of nitrate, indicated that certain crosses produced different amounts of nitrate reductase. Severe drought in an area increased nitrate levels of forages according to Muhrer et al. (1955) and Crawford et al. (1961).

A lack of soil molybdenum has been responsible for nitrate accumulation in higher plants (Spencer and Wood, 1954; Candela et al., 1957; Stout and Meagher, 1948; Hewitt and Jones, 1947). Hewitt and Jones (1947) showed that molybdenum deficient plants do not efficiently reduce nitrate after it has been absorbed and translocated to the upper parts of the plant. Tomato plants accumulated up to 12% of their dry weight as nitrate ion. Forty-eight hours after the addition of molybdenum to these tomato plants, the nitrate ion level was down to 1% and additional

chlorophyll had been formed as shown by the green color of these previously chlorotic plants.

Nitrate in Water Supplies

Walton (1951) found nitrate in drinking water supplies in 17 states. The levels were as high as 440 ppm nitrate and in some cases even higher. Many of the states had reported appreciable levels of methemoglobin and even some deaths of babies due to consumption of this water. Very young babies are unusually susceptible to nitrate poisoning because fetal hemoglobin is more easily oxidized than mature type hemoglobin (Knotek and Schmidt, 1964). Emerick et al. (1965) found that one-week-old pigs injected intravenously with 0.03 gm. sodium nitrite per kg. of body weight developed a lower degree of methemoglobinemia than the same pigs when similarly treated at approximately three months and five and one-half months of age. Seerley et al. (1965) showed that as much as 1330 ppm nitrate or 330 ppm nitrite in water had no effect on the weight gains, thriftiness and reproductive performance of swine. The high concentration of nitrite gave small increases in methemoglobin. There were no effects on liver vitamin A values after 105 days on treatment. Seerley et al. (1965) allowed sheep to drink natural water containing up to 3300 ppm nitrate and observed methemoglobin levels as high as 16% of the total hemoglobin. These high levels of nitrate had no adverse effect on the performance of the lambs.

Metabolism of Nitrate in Ruminants

Nitrate in the rumen is rapidly reduced to nitrite by microorganisms (Wright and Davison, 1964) provided the animal is on a diet containing sufficient molybdenum (Tillman et al., 1965). If the rumen is not

overloaded with nitrate and there is sufficient energy, the nitrate is probably reduced to nitrite and then through hydroxylamine to ammonia. This was reported to be the reaction sequence in soybean leaves, Escherichia coli and Neurospora by Nason (1962). The nitrate ion can be used by the bacteria in two ways; (1) nitrate assimilation and (2) nitrate respiration. Nitrate assimilation represents the "biological reduction of nitrate to ammonia or the amino level with the products being used for the biosynthesis of nitrogen-containing cell constituents, for example, proteins and nucleic acids. The transformation of nitrate to nitrite in the course of nitrate assimilation is the initial step in the enzymatic pathway of the 8-electron change required to attain the oxidation level of nitrogen as represented by the nitrogen of ammonia, amino acids and proteins" (Nason, 1962). Nitrate used in this manner would be a source of nonprotein-nitrogen for the animals. In nitrate respiration, "nitrate is used as the terminal electron acceptor in place of oxygen under anaerobic or partially anaerobic conditions. The reduction products, which may include nitrite, nitric oxide, nitrous oxide, molecular and other oxidation stages of nitrogen, are apparently not further utilized and are for the most part excreted into the surrounding medium" (Nason, 1962).

Muhrer et al. (1955) showed that nitrite was 10 times more toxic to rats and sheep than an equivalent amount of nitrate. This difference is probably due to the animal having time to detoxify the nitrite as it is slowly formed by reduction of the nitrate in the rumen. Both nitrate and nitrite are highly water soluble and are readily absorbed into the bloodstream. Bloomfield et al. (1962a) suggested that nitrate is actively absorbed by the rat stomach. Nitrite, but not nitrate, oxidizes

the ferrous iron of hemoglobin to ferric iron. Nitrate is not reduced to nitrite in the blood (Winter, 1962) and is for the most part excreted in the urine. It has been speculated that the reduction of nitrate to nitrite proceeds at a faster rate than the reduction of nitrite to ammonia and that the excess nitrite is absorbed (Wright and Davison, 1964).

Ruminants are especially susceptible to nitrate toxicity because metabolism and absorption of nitrate and its reduction products occur in the rumen prior to entering the stomach. When massive doses of nitrate are placed directly into the rumen of cattle, it quickly disappears and can be found in large quantities in the blood. But if equal amounts of nitrate are sprayed on hay, the levels of nitrate that appear in the blood are substantially lower (Wright and Davison, 1964). These lower levels of blood nitrate were explained by the slower rate of administration that permitted the animal to reduce more of the nitrate in the rumen or to excrete it via the urine. Once nitrate reaches the blood and is apparently in equilibrium with the extra-cellular fluids (Garner et al., 1961), it is probably metabolized and excreted in a manner similar to that observed in non-ruminants (Kearley et al., 1962). The nitrate is distributed throughout the body and as much as 90% may be recovered in the urine (Wright and Davison, 1964). Setchell and Williams (1962) drenched sheep with nitrate and recovered only 1% to 14% in the urine.

Holtenius (1957) speculated that the vasodilator effect of nitrite might be one aspect of nitrate toxicity in sheep. He found that he gave temporary relief to nitrate poisoned animals when he injected vasoconstrictory drugs. Holtenius (1957) did not measure the blood pressure of these animals but Wright and Davison (1964) found that the blood pressure

of cattle fed 440 to 660 mg. nitrate per kg. of body weight did not differ from control animals. Asbury and Rhode (1964) injected calves with 44 to 66 mg. sodium nitrite per kg. body weight and although there was a small initial drop in blood pressure, they concluded that "the clinical signs of nitrite toxicosis and death were not due to vascular collapse."

Cattle and sheep have been observed to survive the conversion of about 90% of their hemoglobin to methemoglobin (Holtenius, 1957). Holtenius (1957) was unable to demonstrate anoxia in the tissues of sheep when as much as 65% of the hemoglobin was converted to methemoglobin. Also, ruminants adapted themselves to prolonged high levels of nitrate feeding with an increase in their red cell volume and a resultant increase of hemoglobin (Jainudeen et al., 1963; 1964).

Diven et al. (1964) found that pretreatment of sheep with potassium nitrite increased the lethal dose of nitrite by a factor of 1.25 and decreased the amount of methemoglobin formed when given a sublethal dose. This partial immunity was not due to increased hemoglobin levels since the hemoglobin actually decreased from 11 gm. to 10 gm. per 100 ml. blood after four weeks on treatment.

An increase in heart beat may also be a compensatory response of the animal to the lowered oxygen carrying capacity of the blood. Prewitt and Merilan (1958) discovered that the heart beat rate of bull calves rose from 80 beats per minute to 160 beats per minute when methemoglobin rose from 0 to 76% of the hemoglobin. Potassium nitrate had been administered to these calves by gelatin capsule or dissolved in skim milk. Two of the calves were given 33 gm. potassium nitrate per 100 kg. body weight per day for eight days. These calves gained 1 kg. per day and had

less than 10% methemoglobin. Two other calves were given 66 gm. potassium nitrate per 100 kg. body weight per day. These calves had 35% to 50% methemoglobin and did not gain in weight.

It has been suggested that nitrate may interfere with the iodine metabolism of cattle and sheep since it has been shown that nitrate affects iodine metabolism in rats through suppression of thyroxine formation. Wyngaarden et al. (1952) studied the ability of nitrate and nitrite, among other anions, to displace iodide from, or to block iodide uptake by, the thyroid of rats. Although nitrate was the least potent inhibitor of all the anions tested, his data have been cited as evidence that nitrate interferes with thyroid function (Bloomfield et al., 1961, 1962b). Wyngaarden's findings with rats have since been substantiated (Welsch et al., 1961; Yadav et al., 1962; Bloomfield et al., 1961). When nitrate is fed to sheep, it does not have this same blocking effect on iodine. Bloomfield et al. (1961, 1962a) discovered that the thyroid uptake of I^{131} and the concentration of plasma protein-bound I^{131} was increased in sheep fed 0.92% nitrate in their diet. The author has been unable to find experimental reports in the literature to indicate a decreased activity of the thyroid gland of ruminants fed nitrate.

Since an active thyroid had been reported to increase the intestinal absorption and conversion of carotene to vitamin A in rats (Johnson and Bauman, 1957), it seemed that nitrate could exert an effect on vitamin A nutrition through the thyroid. Depletion of liver vitamin A stores has been reported in sheep fed nitrate (Goodrich et al., 1962, 1964; Hatfield et al., 1961) or nitrite (Holst et al., 1961). More recently Ascarelli et al. (1964) showed that there were no adverse effects on carotene utilization when thiouracil was fed to chicks. Numerous other investi-

gators have failed to show that dietary nitrate depleted liver vitamin A stores in cattle or sheep (Hale et al., 1961; Jordan et al., 1963; Weichenthal et al., 1961; Smith et al., 1962; Cline et al., 1962; Sokolowski et al., 1961; Zimmerman et al., 1963; Wallace et al., 1964). In experiments at Cornell (Jainudeen et al., 1965) no thyroid abnormalities were observed in cattle fed nitrate nor was there any indication of impaired vitamin A nutrition. Nitrate has been found to have little or no effect on intestinal destruction or utilization of carotene (Miller et al., 1965; Olson et al., 1963; Davison and Seo, 1963; Davison et al., 1964; Keating et al., 1963). Little et al. (1965) found no effect of nitrate on preintestinal destruction of vitamin A in steers. Thomas (1963) found that nitrate incubated with homogenized rabbit or calf duodenal tissue decreased the amount of vitamin A converted from carotene.

Effects of Nitrate on Growth

Bradley et al. (1940) in an early experiment with long-term feeding of nitrate, concluded that chronic nitrate toxicity did not exist in cattle fed nitrate for more than two months. Clark and Quin (1951) fed diets of "poor quality" grass hay and molasses supplemented with 4% sodium and ammonium nitrate in their search for forms of nitrogen that could be fed to sheep. No toxicity was observed. When 6% and 8% ammonium nitrate or sodium nitrate was offered to sheep, consumption decreased greatly and these diets were discontinued. The addition of 1% to 2% potassium nitrate to corn silage or hay did not reduce gains of cattle nor did additions of 4% potassium nitrate to corn silage affect gains of sheep (Smith et al., 1962). Fattening lambs have been fed up to 4% potassium nitrate in mixed rations high in concentrate with no effect on rate of gain (Cline et al.,

1962, 1963). Nonpregnant and pregnant dairy heifers were fed oat hay containing 1.23% nitrate (Crawford, 1960) and up to 660 mg. nitrate per kg. body weight daily on chopped hay (Davison et al., 1963, 1964) without reduced body weight gains. Work by Wallace et al. (1964) supports the above observations.

A few experiments have been conducted where nitrate feeding has reduced gains. Additions of 1% potassium or sodium nitrate to grain reduced feed consumption and rate of gain of fattening cattle (Hale et al., 1961; Weichenthal et al., 1961). Sokolowski et al. (1961) observed reduced gains when 4% potassium nitrate was mixed in a grain ration for fattening lambs. Although Weichenthal et al. (1963) found that sodium nitrate depressed gains of cattle about ½ kg. daily, liver and plasma vitamin A or carotene were not affected and clinical symptoms of nitrate toxicity were not evident.

From the above reports it would seem that when nitrate is added to roughage no effect on gain is observed unless a coincident decrease in consumption occurs. If nitrate is given in the grain, performance may be adversely affected. This may be attributed to the difference in rate of ingestion of the nitrate.

Effects of Nitrate on Reproduction

There is no doubt that abortions have been caused by nitrates; but the minimum amount that must be consumed cannot be stated precisely. The case histories of abortions cited by Bradley et al. (1940) and Garner (1958) were probably due to a much greater consumption of nitrate than has been described as dangerous to pregnant animals by Muhrer et al. (1956). Simon et al. (1959) produced abortions experimentally by placing about 62 gm. nitrate as potassium nitrate directly into the rumen of pregnant heifers.

Davison et al. (1964) fed 45 dairy heifers nitrate at levels of 0, 440, and 660 mg. per kg. body weight daily beginning three estrous cycles before breeding or at 40, 150, or 240 days of pregnancy continuing until they were killed 30 days after parturition. The estrous cycle remained unchanged, but a decreased conception rate was noted in those fed 660 mg. nitrate per day. One abortion occurred in those fed the lower level of nitrate while two abortions and two deaths occured in those fed the higher level. The gestation periods, the placentas, the birth weight and appearance of the calves, vitamin A and carotene nutrition and milk production were similar for all groups.

Fifteen gm. of potassium nitrate daily as a drench or sprayed on hay given to sheep produced no abortions but reduced birth weight of lambs (Sinclair and Jones, 1964).

Factors Affecting the Severity of Nitrate Poisoning

The rate and quantity of nitrate consumption, energy level or adequacy of the diet, type of forage, adaptation, health, pregnancy and species all affect the susceptibility of an animal to nitrate poisoning. Smaller amounts of nitrate are required to cause death when given suddenly, as by drenching, than when it is incorporated in the diet and thus administered slowly. Also, well fed animals may safely graze forages on which hungry cattle have been poisoned. Hungry cattle eat faster than well fed cattle (Kretschmer, 1958). Sheep and cattle maintained on inadequate diets have been found to be more susceptible to nitrate than those fed adequate diets (Holtenius, 1957; Crawford and Kennedy, 1960). Rumen microorganisms obtained from animals fed alfalfa reduced nitrate and nitrite at faster rates than those obtained from sheep fed grass hays (Sapiro et al., 1949; Pfander et al., 1957). The addition of carbo-

hydrates such as glucose, lactate and similar compounds has been shown to increase the rate of reduction of both nitrate and nitrite by rumen organisms in vitro (Lewis, 1951; Barnett and Bowman, 1957; Emerick et al., 1965). Sapiro et al. (1949) observed that feeding glucose enabled sheep to withstand higher levels of nitrate.

Factors Required for the Complete Reduction of Nitrate

The first step in nitrate reduction is catalyzed by the molybdoflavoprotein nitrate reductase. This enzyme was first characterized from Neurospora (Nason and Evans, 1953) and soybean (Glycine max Merrill) leaves (Evans and Nason, 1953). A similar or closely related nitrate reductase has since been reported in a variety of higher plants (Candela et al., 1957; Hageman and Flesher, 1960; Hageman et al., 1962; Barnett, 1953; Tang and Wu, 1959; Spencer and Wood, 1954). Hewitt and Jones (1947) were the first to suggest that molybdenum was required for the reduction of nitrate in higher plants. Nicholas and Nason (1954) proved that molybdenum is the metal in the Neurospora enzyme when it "(1) increased nitrate reductase activity, (2) decreased during dialysis against cyanide with a concomitant decrease in enzyme activity, (3) reactivated the cyanide dialyzed enzyme and (4) increased the nitrate reductase activity during growth." Molybdenum has been found in nitrate reductase from Agrobacterium tumefaciens (Ramakrishna Karup and Vaidyanathan, 1963) and in E. coli (Taniguchi and Itagaki, 1960). Molybdenum is also required for nitrate reduction by higher plants (Spencer and Wood, 1954; Candela et al., 1957). The following equations illustrate our present knowledge of the mechanism of action of nitrate reductase (Nason, 1962; Nicholas and Stevens, 1955):

$$NADH + H^{+} + FAD^{1} \longrightarrow FADH_2 + NAD$$

$$FADH_2 + Mo^{+6} \longrightarrow Mo^{+5} + FAD$$

$$Mo^{+5} + NO_3^{-} \longrightarrow NO_2^{-} + Mo^{+6}$$

This same sequence and mechanism has also been demonstrated in nitrate reductase from soybean leaves (Nicholas and Nason, 1955a; Evans and Hall, 1955) and Escherichia coli (Nicholas and Nason, 1955b).

Iron (Fewson and Nicholas, 1960) and vitamin K (Medina and Heredia, 1958) have also been shown to be required by E. coli for the reduction of nitrate. The iron requirement is probably due to the need for iron in cytochrome C which is in the electron chain when nitrate is used as a terminal electron acceptor by E. coli (Fewson and Nicholas, 1960). Iron and copper were found to be required for nitrite reduction in the systems employed by Walker and Nicholas (1960), Chung and Najjar (1956) and Nicholas et al. (1960). Lazzarini and Atkinson (1961) could not demonstrate that E. coli needed any cofactors. These workers suggested that the reduction of nitrate to ammonia was catalyzed by a single enzyme and that hydroxylamine was not an obligate intermediate.

Nicholas (1959) suggested that magnesium and manganese were also required for nitrite reduction in higher plants but more recent investigations have not confirmed this (Walker and Nicholas, 1960; Cresswell et al., 1962; Hageman et al., 1962).

Tillman et al. (1965) found that the blood and rumen contents of sheep on a purified diet with no added molybdenum contained significantly more nitrate ion than the blood and rumen contents of sheep on the same

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[1] Flavin adenine dinucleotide (FAD)

diet with 1 ppm molybdenum added. Ten ppm of copper and 100 ppm of iron in the ration had no significant effect on the amounts of nitrite found in the blood.

Adaptive Nature of Nitrate Reductase

Higher plants such as rice (Oryza sativa L.) (Tang and Wu, 1959), cauliflower (Brassica oleracea L. var Botrytis L.), white mustard (Brassica hirta Mvench.) and sunflower (Helianthus annuus L.) (Hewitt and Afridi, 1959) contain the enzyme nitrate reductase in large amounts only when it is required. When these plants are grown with nitrate as the only source of nitrogen, nitrate reductase activity is at its highest level but when ammonium ion is the source of nitrogen, nitrate reductase activity is minimal. Pateman et al. (1964) working with mutants of Aspergillus nidulans which cannot reduce nitrate, presented a theory in which an essential cofactor for nitrate reduction (and xanthine dehydrogenase activity) is also the repressor for nitrate reductase synthesis. They state that "this is the first time that a cofactor seems to also have a regulatory function." The cofactor acts in the normal way to repress nitrate reductase synthesis in the absence of nitrate ion. However, when nitrate is in the medium it combines with the cofactor in such a way as to prevent its "repressor" function. They also found that there is a nitrate induced $NADPH_2$[2]--cytochrome C reductase which, after purifying 70 times, had the same behavior as nitrate reductase. They suggested that these two enzymes were activities of the same protein. The essential features of their theory were as follows:

1. A common cofactor is essential for both nitrate reductase and xanthine dehydrogenase activity in Aspergillus nidulans.

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[2] Reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$)

2. The nitrate reductase protein also possesses $NADPH_2$ linked cytochrome C reductase activity and cofactor is not necessary for this latter function.

3. The presence of cofactor represses the synthesis of the nitrate reductase protein in the absence of nitrate. Nitrate interacts with cofactor in such a way as to prevent its "repressor" function.

More recent work by Sorger (1965) supports this single enzyme theory. Sorger (1965) suggests that the enzyme is an aggregate of two polypeptides; one transports electrons from NADH to FAD to cytochrome C and the other accepts electrons from reduced FAD in the first polypeptide, passes them to molybdate and from there to nitrate.

Nitrate Reductase Activity in Animal Tissues

The ability of animal tissues to reduce nitrate was first demonstrated in liver preparations of various species by Bernheim and Dixon (1928). They showed that nitrate was enzymatically reduced by functioning as a hydrogen acceptor of aldehyde oxidase instead of oxygen. However, the more recent studies of Omura (1959) and Omura and Takahashi (1959) have led to the suggestion that the nitrate reductase activity of animal cell preparations may not necessarily be due to the aldehyde oxidase and xanthine oxidase activities. Walters and Taylor (1964, 1965) have found that minced fresh pig muscle will reduce nitrite to gaseous nitric oxide which indicates that nitrite is able to compete with other electron acceptors for the reductive processes of mammalian respiratory enzyme systems.

CHAPTER III

ANALYSIS OF FLORIDA WATER FOR NITRATE

Water has been shown to contain appreciable amounts of nitrate (Walton, 1951). A survey of some of the rivers, lakes and wells of Florida was undertaken to determine if they were sufficiently high in nitrate to cause a toxicity problem in the state.

Experimental procedure

Water samples were collected in polyethylene bottles from 22 locations in Central Florida. The samples were stored at 5°C. until they were analyzed for nitrate and nitrite (A. P. H. A., 1960).

Results and discussion

The results of the above analyses are shown in Table 1. Metzler and Stoltenberg (1950) concluded that water containing 10 to 20 ppm nitrate was unsafe for infants. Most of the samples listed in Table 1 were relatively low in nitrate but the sample from the well at the corral at the Everglades Correctional Institution (E. C. I.) and the Hillsborough River sample may have been high enough in nitrates to have had some subclinical effect on human infants. These levels of nitrate in water would probably have had no adverse effect on sheep or swine (Seerley *et al.*, 1965). Two of the samples obtained from Belle Glade contained relatively high levels of nitrite (Well TR 859 and canal beside office) which could

TABLE 1

CHLORINE, NITRATE AND NITRITE OF WATER SAMPLES FROM CENTRAL FLORIDA

Location	Cl^- (ppm)	NO_3^- (ppm)	NO_2^- (ppb)
St. Johns' River, Sanford	272	3.7	16.5
St. Johns' River, Palatka	226	2.2	trace
Kissimmee River, Okeechobee	28	0.9	16.5
Lake Tohopekaliga	-	0.7	15.7
Small unnamed river at Junction U.S. 441 and St. 60	16	0.6	trace
Fisheating Creek, Palmdale	23	0.6	trace
Taylor Creek at Rt. 441	112	1.9	7.5
Canal near office, E.E.S., Belle Glade	209	2.8	153.0
Peace River, Zolfo Springs	17	1.8	3.4
Paynes Prairie	24	0.8	5.5
Well near corral, R.C.E.S., Ona	24	0.7	trace
Orange Lake, McIntosh	17	1.0	2.0
Canal by corral, E. C. I., Belle Glade	212	1.3	10.5
Dr. Haines' well, at home, Belle Glade	77	1.4	7.5
Well TR 852, E. C. I., Belle Glade	228	1.1	7.5
Well TR 859, E. C. I., Belle Glade	169	0.4	610.0
Well at corral, E. C. I., Belle Glade	529	4.8	73.5
Caloosahatchee River, Moore Haven	75	1.4	16.5
Lake Harris, Leesburg	14	1.4	0
Lake Okeechobee, Clewiston	64	1.9	5.5
Hillsborough River, Tampa	258	5.9	76.5
Withlacoochee River, Dunnellon	15	1.9	12.5

have been potentially harmful if used as a source of drinking water for cattle or humans.

Since knowledge of the chloride content of these water samples was a prerequisite to analyzing them for nitrate, the chloride values are given in Table 1. Several of the samples were high in chloride and the water from the well at the corral, E. C. I., was quite salty to the taste.

CHAPTER IV

THE EFFECT OF A NITRATE DRENCH ON SHEEP CONSUMING A PURIFIED DIET

Sosa (1964) at the University of Florida fed yearling native ewes a purified diet containing 2% sodium nitrate. The purpose of the nitrate was to accelerate a vitamin A deficiency. These ewes were continued on the purified diet and blood methemoglobin values were determined by the author. The sheep then had the nitrate deleted from their diets and equivalent amounts of nitrate were given daily as a drench. This was done to compare the effect of two types of oral nitrate intake on the rate and extent of methemoglobin formation in sheep.

Experimental procedure

Trial 1.--Six Florida native ewes approximately one year of age were fed the purified diet given in Table 2. Blood from the jugular vein was obtained nine times during a period of two months and analyzed immediately each time for methemoglobin by the method of Evelyn and Malloy (1938).

Trial 2.--The six sheep used in Trial 1 were fed the same purified diets except nitrate was deleted in Trial 2. After one month on feed, the sheep were drenched with 16.8 gm. of nitrate as the sodium salt at 9:00 a.m. Blood samples were obtained just prior to drenching and at approximately 3-hour intervals during the day. The samples were immediately analyzed for methemoglobin. The surviving sheep were again drenched and sampled for six consecutive days.

TABLE 2

COMPOSITION OF EXPERIMENTAL RATION

Ingredient	Percent
Cellulose (Solka-floc)	20
Casein (90% protein)	20
Corn Starch	23
Corn Sugar	23
Corn Oil	4
$NaNO_3$	2
Trace Mineral Premix B	0.5
Minerals A	6.5
Vitamin Premix C	1.0
	100.0

A. Minerals	Gm./100 kg. Feed
$CaHPO_4.2H_2O$	3,177
$K_2CO_5.1\frac{1}{2}H_2O$	2,047
$MgSO_4.3H_2O$	697
NaCl	481

B. Mineral Premix[a]	Gm./100 kg. Feed
$FeSO_4.2H_2O$	87.6
$Na_2B_4O_7.10H_2O$	12.3
$MnSO_4.H_2O$	6.4
$ZnSO_4.7H_2O$	17.6
KI	1.9
$CuSO_4$	2.6
$2CoCO_3.Co(OH)_2$	0.035
MoO_3	0.051

C. Vitamin Premix[b]	Gm./1000 kg. Feed
Vitamin A (250,000 I. U./gm.)[c]	26.4
Vitamin D (3,000 I. U./gm.)	220.0
Vitamin E (100,000 I. U./454 gm.)	176.0
Choline Chloride (25% choline)	4,400.0
Total	4,822.4

[a] One-half kg. mixed with each 100 kg. feed.
[b] One kg. mixed with each 100 kg. feed.
[c] Add 24 gm. of corn sugar to the vitamin A deprived ration.

Trial 3.--Trial 3 was conducted to provide more frequent sampling and hemoglobin values. Four more sheep were obtained that had been on the purified diet containing no nitrate or vitamin A for two months. These four sheep, in addition to the two sheep that survived Trial 2, were drenched with 16.8 gm. of nitrate as the sodium salt and blood samples were obtained hourly. Hemoglobin (Hawk et al., 1954) and methemoglobin levels were immediately determined. The sheep that survived were drenched and sampled for five consecutive days.

Trial 4.--The five sheep that survived Trial 3 were drenched with 33.6 gm. of nitrate and were bled hourly. This amount of nitrate was twice as much as they had received in Trials 2 and 3. Hourly blood samples were obtained. Three sheep died the first day and the two remaining sheep were drenched with 33.6 gm. of nitrate the following day.

Results and discussion

Trial 1.--The results of Trial 1 are shown in Table 3. Detectable levels of methemoglobin were observed in only four of the six sheep.

TABLE 3
METHEMOGLOBIN OF SHEEP FED 2% SODIUM NITRATE IN DIET

Sheep No.	Date Sample Was Obtained in 1963								
	3/26	3/27	3/29	4/3	4/11	4/15	4/24	5/11	5/22
94	0[a]	0	0	0	0	0	0	0	0
93	0	0	0	0	0	0	0.3	0	0
86	0	0	0	0.3	0	0	0	0	0
87	0	0	0	0	0	0.3	0	0	0
96	0	0	0	0	0	0	0	0	0
W75	0	0.3	0.2	0.2	0	0	0	0	0

[a] Gm. per 100 ml. blood.

Sinclair and Jones (1964) found that sheep that consumed hay on which potassium nitrate had been sprayed to a final level of 0.93% nitrate had no methemoglobin initially or after several months. Holst et al. (1961) fed wethers mixed feed which contained from 0.1% to 0.75% nitrite for several months but no methemoglobin was found in the blood of these sheep.

Feeding ewes approximately 17 gm. of nitrate per day did not precipitate nitrate poisoning symptoms or even raise blood methemoglobin levels very high. These ewes were then drenched with the same amounts of nitrate that they had consumed daily in their feed and their responses to this type of dosage were studied.

Trial 2.--The results of Trial 2 are shown in Table 4. The sheep that died were examined by Dr. F. C. Neal of the Department of Veterinary Science and the cause of death was given as nitrate poisoning in every case.

The results of drenching ewes with 16.8 gm. of nitrate were much more drastic (Table 4) than when the same amount of nitrate was consumed in the feed (Table 3). This was probably due to the faster rate of intake when the nitrate was given as a drench. With a drench, the rumen bacteria do not have time to reduce the nitrate beyond the highly toxic nitrite form. When the animal is drenched with nitrate the initial reduction of nitrate to nitrite proceeds at a faster rate than the reduction of nitrite to ammonia and nitrite may accumulate and be absorbed by the blood (Wright and Davison, 1964).

Trial 3.--The results of Trial 3 are shown in Table 5. The first day of drenching there was little methemoglobin formed. The second day sheep 86 and 92 had over half of their hemoglobin converted to methemo-

TABLE 4

METHEMOGLOBIN IN SHEEP DRENCHED WITH 16.8 gm. NITRATE (TRIAL 2)

Sheep No. and Date	Time After Drenching			
7/23/63	0 Hour	2 Hours	4 Hours	8½ Hours
94	0[a]	0	-	-
93	0	2.5	(died at about 5 hours)	
86	0	0	-	-
87	0	0	-	-
96	0	0.4	-	-
W75	0.4	0	-	-
7/24/63				
94	0	1.4	4.1	(died at about 5 hours)
86	0	0	0	-
87	0	0	1.4	(died the following night)
96	0	0.2	0	-
W75	0	0	0	-
7/25/63				
86	0	0	0	-
96	0	0	0	-
W75	0	0.5	2.5	-
7/26/63				
86	0	0	1.1	-
96	0	0	0	-
W75	0	1.6	5.9	(died at 5½ hours)
7/27/63				
86	0	-	1.7	3.9
96	0	-	0	0.8
7/28/63				
86	0	-	1.2	-
96	0	-	0.3	-

[a] Gm. per 100 ml. blood

TABLE 5

METHEMOGLOBIN IN SHEEP DRENCHED WITH 16.8 gm. NITRATE (TRIAL 3)

Sheep No. and Date	Time After Drenching											
	0 Hours		3 Hours		6 Hours		9 Hours		12 Hours		15 Hours	
8/12/63	gm.[a]	%[b]	gm.	%	gm.	%	gm.	%	gm.	%	gm.	%
86	0	0	0	0	0.9	9.4	0.4	3.2	-	-	-	-
88	0	0	0	0	0	0	0	0	-	-	-	-
89	0	0	0	0	0	0	0	0	-	-	-	-
90	0	0	0	0	0	0	0	0	-	-	-	-
92	0	0	0	0	0	0	0.6	6.5	-	-	-	-
96	0	0	0	0	0	0	0	0	-	-	-	-
8/13/63												
86	0	0	1.1	11.1	4.6	40.8	7.0	56.4	6.4	59.6	-	-
88	0	0	0	0	0.4	3.1	1.0	7.5	0.4	2.8	-	-
89	0	0	0.5	4.5	0.7	6.0	0	0	-	-	-	-
90	0	0	0	0	0	0	0	0	-	-	-	-
92	0.4	2.8	0.5	4.3	3.4	27.3	6.5	52.4	5.8	46.9	-	-
96	0	0	0	0	1.0	10.4	0	0	-	-	-	-
8/14/63												
86	0	0	0.8	8.4	1.3	12.3	1.7	15.1	2.4	20.1	0.7	6.7
88	0	0	0.7	5.4	0.5	3.8	0.5	4.1	0	0	-	-
89	0	0	1.4	12.2	3.2	27.7	3.3	26.3	0.5	4.8	-	-
90	0	0	0	0	0.2	2.1	0	0	0	0	-	-
92	0.4	2.8	0.7	5.8	1.9	15.3	4.2	33.2	3.0	21.8	0.7	5.8
96	0	0	3.0	32.4	5.0	49.2	2.5	24.9	0.8	7.4	-	-
8/15/63												
86	0	0	1.1	9.4	4.0	33.6	6.9	56.3	5.8	47.5	-	-
88	0	0	1.1	10.1	1.6	12.9	1.8	13.6	0.5	4.2	-	-
89	0	0	0.9	8.2	3.0	24.7	2.2	19.7	0.6	5.6	-	-
90	0	0	0.5	4.2	0	0	0	0	-	-	-	-
92	0	0	0.6	5.0	1.8	13.8	0.8	7.0	0.4	3.0	-	-
96	0.2	2.3	4.4	45.2	5.9	58.7	(at death)					
8/16/63												
86	0.2	2.4	1.1	10.2	3.6	31.6	5.7	44.6	2.5	23.2	0.6	5.8
88	0	0	2.8	23.3	5.6	39.4	6.4	44.5	4.6	35.7	0.9	7.7
89	0	0	1.2	10.4	3.4	32.8	5.3	44.4	1.4	13.0	0	0
90	0	0	0.3	2.5	0.9	8.5	0.6	5.6	0	0	-	-
92	0	0	1.2	10.3	5.3	46.1	2.8	22.3	0.9	8.0	0	0

[a] Gm. methemoglobin per 100 ml. blood.

[b] Methemoglobin as percent of hemoglobin.

globin while the other sheep had only small amounts or none converted. The third day sheep 86, 88 and 92 did not reach the high levels of methemoglobin attained on day 2, but the other three sheep had higher values. On day 4 the methemoglobin values were about the same as on previous days except for number 96 which died five hours after drenching. It had 58.7% methemoglobin at the time of death.

The fifth day of drenching, sheep 86 did not reach as high a level of methemoglobin as on the fourth day but the other sheep had more than double the methemoglobin levels of the day before. Overall, the sheep that survived regenerated their hemoglobin by the next morning. However, each day it was apparent that more methemoglobin was formed, which indicated that the sheep gradually lost their capacity to resist the toxic nitrate. Sokolowski et al. (1960) suggested that lambs were apparently able to counteract nitrate toxicity for about 48 hours due to detoxification by specific microbes with the eventual elimination of these microbes. Another explanation of the increasing daily levels of methemoglobin would be an impaired ability of the blood and tissues either to reduce nitrite or to eliminate it via the urine.

Trial 4.--The results of Trial 4 are shown in Table 6. Three of the five sheep died the first day. Of the two remaining sheep, one died the second day. The other, at the terminal stage of nitrate poisoning, was administered intravenously a 3% solution of methylene blue and it recovered. The amount of hemoglobin converted to methemoglobin at the time of death varied between 51.5% and 70.6%. This is quite a wide range and is lower than some of the high values reported in the literature (Holtenius, 1957; Diven et al., 1964; Setchell and Williams, 1962 and Stewart and Merilan, 1958). Since Diven et al. (1964) found 93%

TABLE 6

METHEMOGLOBIN OF SHEEP DRENCHED WITH 33.6 gm. NITRATE (TRIAL 4)

Sheep No. 8/19/63	0 Hour gm.[a]	0 Hour %[b]	1 Hour gm.	1 Hour %	2 Hours gm.	2 Hours %	3 Hours gm.	3 Hours %	4 Hours gm.	4 Hours %	5 Hours gm.	5 Hours %	6 Hours gm.	6 Hours %	7 Hours gm.	7 Hours %
	Hours after Drenching															
86	0	0	0.7	5.7	2.1	18.9	5.6	44.1	6.2	49.4	7.7	62.7	6.9	54.3	(at death)	
88	0	0	0.5	4.5	1.8	15.1	2.5	19.1	5.0	40.7	5.0	45.2	4.8	37.6	4.6	35.1
89	0	0	0	0	0	0	0	0	0	0	0.9	8.4	2.3	20.0	3.9	31.1
90	0	0	0	0	0	0	0	0	0	0	0.4	3.5	0.8	7.5	1.7	15.2
92	0	0	0.6	4.9	0.9	7.6	1.6	12.7	2.8	22.6	4.6	57.8	5.4	47.6	6.1	51.5
															(at death)	

Sheep No. 8/19/63	8 Hours gm.	8 Hours %	9 Hours gm.	9 Hours %	10 Hours gm.	10 Hours %	11 Hours gm.	11 Hours %	12 Hours gm.	12 Hours %	13 Hours gm.	13 Hours %	14 Hours gm.	14 Hours %	15 Hours gm.	15 Hours %
	Hours after Drenching															
88	4.9	38.7	4.5	34.3	4.1	45.7	3.8	36.4	3.5	27.4	3.3	25.2	3.2	24.5	2.6	23.0
89	5.9	45.7	7.2	60.6	(at death)											
90	2.4	22.3	2.9	24.5	3.0	25.0	2.6	23.8	1.8	16.7	1.5	13.8	1.2	11.0	1.0	11.0

Sheep No. 8/20/63	0 Hours gm.	0 Hours %	1 Hour gm.	1 Hour %	2 Hours gm.	2 Hours %	3 Hours gm.	3 Hours %	4 Hours gm.	4 Hours %	5 Hours gm.	5 Hours %	6 Hours gm.	6 Hours %	7 Hours gm.	7 Hours %
	Hours after Drenching															
88	0	0	0.5	4.9	1.5	13.7	2.8	23.2	3.9	32.6	5.1	39.6	5.2	48.0	6.1	51.1
90	0	0	0	0	0.4	4.6	1.0	9.9	1.8	18.9	2.5	23.1	3.8	33.8	5.3	46.7

Sheep No. 8/20/63	8 Hours gm.	8 Hours %	9 Hours gm.	9 Hours %	10 Hours gm.	10 Hours %	11 Hours gm.	11 Hours %
	Hours after Drenching							
88	7.3	58.6	6.0	48.0[c]	2.9	25.8	3.4	29.5
90	5.6	49.7	7.3	70.6	(at death)			

[a] Gm. methemoglobin per 100 ml. blood.
[b] Methemoglobin as percent of hemoglobin.
[c] A 3% solution of methylene blue administered intravenously.

methemoglobin in sheep, death of the sheep reported herein at comparatively low levels of methemoglobin may have been caused by the unusual amount of handling associated with drenching and frequent bleeding.

There seems to have been no immunity imparted by feeding nitrate as suggested by Diven et al. (1964) since four of the sheep that had been fed nitrate previously died during Trial 2 and only one of the sheep that had not previously been fed nitrate died during Trial 3. The sheep that had been fed nitrate previously were not fed the nitrate for approximately one month prior to nitrate drenching, thus any immunity from previous treatment may have been lost during this time. If these sheep had had more red blood cells due to nitrate feeding as suggested by Jainudeen et al. (1963), the red blood cell volume could have adjusted to normal during the one month preliminary period (Jamieson, 1958).

These trials with purified diets and nitrate feeding and drenching demonstrated the susceptibility of sheep to nitrate poisoning. However, it was believed that studies involving the influence of molybdenum and copper on nitrate toxicity should be done using natural forage rations containing various concentrations of molybdenum and copper and high levels of nitrate.

CHAPTER V

SUPPLEMENTARY MOLYBDENUM AND THE REDUCTION OF NITRATE IN THE RUMEN OF STEERS FED MILLET SOILAGE CONTAINING HIGH LEVELS OF NITRATE

In the spring of 1964 the University of Florida Dairy Research Unit, Hague, had several fields of millet (Pennisetum glaucum L.) which contained between 2% and 4% nitrate on the dry matter basis. This millet was chopped daily and fed to the milking herd, dry cows, springing heifers and to some two-year-old dairy steers. The high levels of nitrate had no observable adverse effects on these animals. Normally all of the animals at the Dairy Research Unit are fed minerals. However, the dairy steers had inadvertently received no minerals for several months. It was thought that if these steers were given molybdenum, the nitrate would be reduced to nitrite at a faster rate than the nitrite could be reduced and nitrate poisoning symptoms would be detected.

Experimental procedure

Two-year-old Guernsey and Jersey steers were assigned to the treatments presented in Table 7. Two steers were fed alfalfa hay with added molybdenum to check on the possible toxicity of molybdenum. The two steers on each treatment were fed in open lots and observed several times daily for signs of nitrate poisoning. Blood samples were obtained the eighth day on feed and analyzed for methemoglobin.

After three weeks on treatment, one steer receiving millet plus molybdenum and one steer receiving millet with no supplementary molybdenum

were allowed to eat millet for 30 minutes. They were then drenched with 62 gm. nitrate as sodium nitrate. Rumen samples were obtained periodically by stomach tube for three hours and analyzed for nitrate.

TABLE 7

DESIGN FOR TESTING INFLUENCE OF MOLYBDENUM ON NITRATE REDUCTION IN THE RUMEN OF STEERS EATING MILLET WITH HIGH LEVELS OF NITRATE

Steer No.	Treatment
486, 487	High nitrate millet plus 0.5 gm. $NaMoO_4 \cdot 2H_2O$[a]
912, 933	High nitrate millet
80, 256	Alfalfa hay plus 0.5 gm. $NaMoO_4 \cdot 2H_2O$

[a] The 0.5 gm. of $NaMoO_4 \cdot 2H_2O$ was mixed in a handful of citrus pulp and fed every other day.

Results and discussion

No toxicity symptoms were observed in the steers during the first eight days of the experiment. Blood samples were obtained on the eighth day and analyzed for methemoglobin (Table 8). The steers consuming the high-nitrate millet had very low levels of blood methemoglobin while those receiving the alfalfa had no methemoglobin. Nothing conclusive could be said concerning the molybdenum treatment.

When nitrate was given as a drench, the steer that received no supplementary molybdenum had higher levels of nitrate throughout the sampling period as shown in Figure 1. These preliminary studies suggested that molybdenum may have been responsible for an increased rate of nitrate disappearance from the rumen.

TABLE 8

METHEMOGLOBIN IN STEERS FED HIGH NITRATE MILLET SOILAGE WITH AND WITHOUT MOLYBDENUM

Steer	Treatment	Methemoglobin[a]
486	Millet + Mo	0.7
487	Millet + Mo	0.1
912	Millet	0.3
933	Millet	1.1
80	Alfalfa + Mo	0
256	Alfalfa + Mo	0

[a] Gm. per 100 ml. blood.

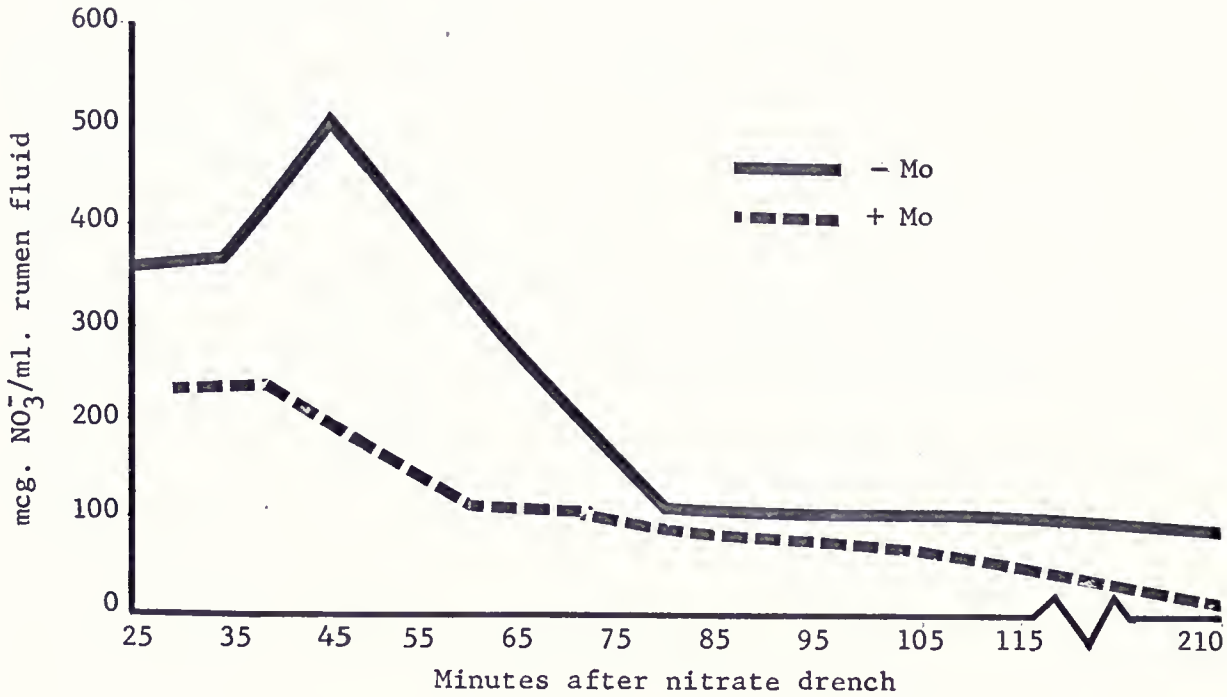

Figure 1. Rate of Nitrate Disappearance From Rumen Fluid of Steers Drenched With 62 gm. of Nitrate 30 Minutes After Feeding.

CHAPTER VI

THE EFFECT OF MILLET GROWN WITH HIGH LEVELS OF NITRATE, WITH AND WITHOUT MOLYBDENUM AND COPPER, WHEN FED TO SHEEP AND CATTLE

In order to determine the effect of varying concentrations of molybdenum and copper in forage on the rate of nitrate reduction in the rumen, the concentrations of nitrate and ammonia in rumen contents and methemoglobin and ammonia in blood were determined. The pH, carbon dioxide and volatile fatty acid changes in the rumen were also observed.

Star millet was raised in 1964 with high levels of nitrogen fertilizer, with and without added molybdenum and copper. This was an effort to produce forage with high levels of nitrate, molybdenum and copper. The forage was irrigated when required, harvested, chopped and dried at the pre-bud stage of maturity. The forage was fed to yearling wethers.

Gahi millet was raised in 1965 and harvested in approximately the same manner as that in 1964. This forage was fed yearling Hereford steers in a 4x4 Latin Square experiment. The steers were equipped with rumen fistulas.

Experimental procedure

Trial 1 (1964).--On April 1, 1964 Star millet was sowed and fertilized at the rate of 198 kg. per acre with 8-8-8 fertilizer. The field was divided into four plots and the following applications were made: Plot A, no supplemental copper or molybdenum; Plot B, 11.35 kg. copper sulfate per acre at planting and 11.35 kg. copper sulfate per acre on

June 2; Plot C, 0.91 kg. sodium molybdate at planting and 0.91 kg. sodium molybdate on June 2; Plot D, both copper and molybdenum at the rates given for Plots B and C above. The supplemental copper and molybdenum were carefully weighed and mixed with the fertilizer applied to each plot. All four plots were top-dressed on April 27 at the rate of 173 kg. ammonium nitrate per acre. The plots were again top-dressed on May 5 and June 2 at the same rate for a total of 189 kg. of actual nitrogen per acre for the season. The plots were irrigated when necessary.

The millet was cut May 19 and the forage was removed but was not used experimentally. The second cutting of millet was made at the pre-bud stage of maturity with a forage harvester on June 9. The chopped forage was loosely bagged in large loose-weave burlap bags and dried artificially at Mixons' Crop Drying Service, Williston, Florida. The analysis of the second-cut forage is shown in Table 9.

TABLE 9

COMPOSITION OF MILLET, 1964

Plot (Treatment)	NO_3(%)	NO_3-N(%)	Crude Protein(%)	Cu(ppm)	Mo(ppm)	Inorganic Sulfate(%)
A (-Cu, -Mo)	1.76	0.40	23.3	4.2	1.68	0.32
B (+Cu, -Mo)	1.95	0.44	24.1	78.3	0.56	0.40
C (-Cu, +Mo)	2.11	0.48	23.9	5.2	17.68	0.46
D (+Cu, +Mo)	1.64	0.37	24.2	98.7	27.26	0.35

Nitrate content was determined by the method of Woolley et al. (1960), molybdenum and copper by methods given by Sandell (1959) and

sulfate by Steinbergs' (1953) method. Crude protein was determined by Kjeldahl method (A.O.A.C., 1960).

Sulfate was not an experimental variable and its concentration in the forages was relatively uniform. The high levels of copper obtained in Treatments B and D were probably due to contamination with the second application of copper sulfate which was applied one week before harvest. The millet was irrigated two times with overhead sprinklers between the last fertilization and cutting but the supplemental copper could have been trapped in the swirls of the millet plants. The same conditions may have been true for the molybdenum.

The millet grown in 1964 was fed, along with water ad libitum, to 24 yearling wether sheep which weighed from 23 to 38 kg. The sheep were housed in 4' x 8' pens with three sheep per pen and were randomly allotted to treatments with six sheep per treatment. Each treatment forage was fed continuously to each group throughout the two month duration of the trial. Two days after the trial began, one wether on Treatment B accidentally strangled and two wethers on Treatment A died due to unknown causes. After one month on feed jugular blood and rumen samples were taken from one animal on each treatment at 8:00 a.m. Feed had been withheld the previous night. The sheep were then allowed access to feed for one hour and blood and rumen samples were obtained. The sheep were then drenched with 20 gm. potassium nitrate as a 20% solution which was approximately 0.66 gm. per kg. body weight. Rumen contents were sampled, with a stomach tube, at the following intervals after drenching: 10, 30, 50, 70, 90 minutes, 2, 3, 4 and 6 hours. Blood samples were taken at the following intervals after drenching: 1, 2, 3, 4 and 6 hours. The above sampling

procedure was repeated once a week until four sheep from each treatment had been sampled.

The pH of the rumen samples was lowered to 1 to 1.5 with concentrated hydrochloric acid. They were stored frozen until analyzed for ammonia by the microdiffusion method of Conway (1957), after which the samples were filtered through analytical filter aid (Johns-Manville, Celite) with vacuum and analyzed for nitrate by the method of Woolley et al. (1960).

The blood samples were collected in lithium citrate to prevent clotting and analyzed for ammonia (Conway, 1957) and methemoglobin (Evelyn and Malloy, 1938).

Trial 2 (1965).--On April 6, 1965, Gahi millet was planted in a field at the Dairy Research Unit, Hague, which had been cleared only five years before and had purposely not been fertilized with trace minerals. Three hundred and thirty-six kg. of 10-10-10 fertilizer per acre were applied at planting time. The field was divided into four equal-sized plots and the following applications were made: Plot A, no supplemental copper or molybdenum; Plot B, 12.5 kg. copper sulfate per acre; Plot C, 1 kg. sodium molybdate per acre; Plot D, both copper and molybdenum at the rates given for Plots B and C above. All four plots were top-dressed with 45.4 kg. nitrogen per acre as liquid ammonia on April 28 and with 200 kg. ammonium nitrate per acre on May 12 for a total of 146 kg. actual nitrogen per acre for the season. 368 Kg N / ha for season

The millet was harvested on May 21 and made into hay in the same manner as described for 1964. Table 10 shows the analysis of this forage.

TABLE 10 - See table 13, page 46

COMPOSITION OF MILLET, 1965

Plot (Treatment)	NO_3(%)	NO_3-N(%)	Crude Protein(%)	Cu(ppm)	Mo(ppm)	Inorganic Sulfate(%)
A (-Cu, -Mo)	0.75	0.17	11.5	13.74	0.58	0.21
B (+Cu, -Mo)	0.39	0.09	12.7	11.71	0.57	0.21
C (-Cu, +Mo)	0.33	0.08	13.2	9.17	3.00	0.16
D (+Cu, +Mo)	0.49	0.11	13.2	10.66	4.34	0.32

The millet grown in 1965 was fed to four rumen-fistulated yearling Hereford steers in a 4x4 Latin Square. The steers were stabled in individual pens eight feet square with an 8' x 20' exercise yard. Water was supplied in automatic drinking cups. The hay was offered to the steers for one hour at 8:00 a.m. and for one hour at 4:00 p.m. It was thought that by allowing the steers only a limited time to eat each day, that they would eat faster and get the nitrate of the millet into the rumen as quickly as possible. There should have been a greater chance of observing nitrate poisoning symptoms under these conditions.

There was a 14 day preliminary feeding period before each collection. Rumen pumps (Moore et al., 1964) were installed in the fistulas the evening of the 14th day and the drinking water was removed. The morning of the 15th day rumen samples were obtained from each steer. This was designated the 0-time sample. Feed and water were offered for one hour. Rumen samples were then obtained at the following intervals: 1, 1.33, 1.67, 2, 2.5, 3, 4, 5, 6, 7 and 8 hours. The pH of the rumen samples was determined immediately. An aliquot was preserved by adding

concentrated hydrochloric acid to pH 1 to 1.5 and frozen for nitrate (Woolley et al., 1960) and ammonia (Conway, 1957) analysis. Another aliquot was preserved with mercuric chloride and frozen for volatile fatty acid determination. Volatile fatty acids were analyzed by gas-liquid chromatography. Additional samples were placed in serum bottles, quickly stoppered and immediately analyzed for total rumen fluid carbon dioxide concentration (bicarbonate plus carbonic acid-carbon dioxide, Moore et al., 1964).

Results and discussion

Trial 1 (1964).-- Results of the analysis of samples taken from the sheep are shown in Table 11. There was so much individual variation within treatments that no trends for a different rate of disappearance of nitrate from the rumen could be detected in any of the treatments. Levels of rumen ammonia were not different among treatments (Table 16). The concentrations ranged between 10 and 30 mg. of ammonia nitrogen per 100 ml. rumen fluid which is within a range considered normal for animals on good-quality hay by Barnett and Reid (1961). Although there were relatively high levels of protein and 1.6% to 2.1% nitrate in the hay, both of which would be potential sources of rumen ammonia, the ammonia concentrations were not extraordinarily high.

There were only very small differences among treatments in blood ammonia. The range was between 0.30 and 1.1 mg. of ammonia nitrogen per 100 ml. blood serum. This is within a normal range for blood ammonia (Barnett and Reid, 1961).

Two sheep, one on Treatment B and one on Treatment C, collapsed about four hours after drenching and were treated with methylene blue.

TABLE 11

THE AMMONIA AND NITRATE OF RUMEN FLUID AND THE BLOOD AMMONIA AND METHEMOGLOBIN OF SHEEP, 1964

Time	Prefed	Predrench	10 min.	30 min.	50 min.	70 min.	90 min.	2 hrs.	3 hrs.	4 hrs.	6 hrs.
				Rumen NH_3 (mg. NH_3-N/100 ml. rumen fluid)							
Treatment											
A	11.3[a]	24.5	18.4	22.5	18.7	19.7	22.0	27.2	25.2	19.7	18.9
B	11.7	27.4	18.5	21.7	20.1	17.1	25.6	27.6	30.8	23.4	27.6
C	12.5	17.0	16.2	18.6	17.0	16.8	20.6	17.9	19.2	21.1	20.4
D	12.5	22.4	20.7	23.9	22.2	21.1	23.0	19.2	21.6	21.8	18.5
				Rumen NO_3 (mcg. NO_3/ml. rumen fluid)							
Treatment											
A	12.4	14.2	1439	1315	651	399	430	313	138	103	55
B	15.9	73.1	1218	1373	1129	576	961	160	75	44	97
C	26.6	17.7	886	1638	952	1373	731	345	142	260	185
D	6.6	57.6	1506	1329	1010	288	496	421	461	182	9
				Blood NH_3 (mg. NH_3-N/100 ml. serum)							
Treatment											
A	0.56	0.57	-	0.56	0.56	-	0.80	0.46	0.48	0.45	0.53
B	0.71	0.45	-	0.54	0.51	-	1.00	0.48	0.49	0.51	0.49
C	0.54	0.62	-	0.58	0.54	-	0.72	0.47	0.48	0.51	0.58
D	0.79	0.63	-	0.58	0.48	-	1.10	0.46	0.44	0.45	0.30
				Blood methemoglobin (gm. MHb/100 ml. blood)							
Treatment											
A	1	0	-	0	0.20	-	0.18	0.81	2.53	2.44	1.91
B	0.06	0	-	0	0.36	-	1.1	1.23	2.82	3.77	2.74
C	0	0	-	0	0.27	-	0.61	1.68	2.20	3.40	1.28
D	0.06	0	-	0	0	-	0	1.32	1.82	2.09	3.41

[a] Averaged values.

The sheep on Treatment B died a few minutes later; the sheep on Treatment C survived. Since methylene blue had been administered, meaningful methemoglobin values were not obtained.

Blood methemoglobin values gradually increased and reached a maximum about four hours after the nitrate dose. This was several hours earlier than when the highest level of methemoglobin was observed in the sheep fed purified diets (Chapter IV). The most extreme toxicity symptoms, due to a potassium nitrate drench to lambs, were observed at 4½ hours by Sokolowski et al. (1960). Lewis (1951) observed a peak in methemoglobin values seven hours after a lamb was dosed with 25 gm. of sodium nitrate. The period of time required for the peak levels of methemoglobin to appear is apparently quite variable and is probably due to many factors including the amount of nitrate ingested, the carbohydrate levels of the ration and immediate history of nitrate ingestion. A possible explanation of the difference in time required to reach maximum methemoglobin concentrations among the sheep reported in this research would be the amount of carbohydrates in the diet. The purified diets fed to sheep reported in Chapter IV contained high levels of fermentable carbohydrates (Table 2) but the sheep in this trial (1964) received millet hay with no grain.

Trial 2 (1965).--The averaged results of analysis of the samples taken from the steers in Trial 2 are shown in Table 21. Individual values are given in Tables 17 through 20 and 26. Steers that consumed the four forage treatments in 1965 showed no statistical differences in rumen nitrate levels four hours after they had eaten the forage. There were also no statistical differences at four hours in micrograms of nitrate per 5 ml. rumen fluid per gm. of nitrate fed. There were no differences in the rate of nitrate reduction due to treatment

TABLE 12

THE pH, NITRATE, AMMONIA AND CARBON DIOXIDE CONTENT OF RUMEN FLUID FROM STEERS, 1965

Time, hrs.[a]	0	1	1.33	1.67	2	2.50	3	4	5	6	7	8
						pH Rumen Fluid						
Treatment												
A	-	6.26[b]	6.15	6.15	6.25	6.37	6.23	6.30	6.18	6.20	6.22	6.41
B	-	6.36	6.30	6.25	6.35	6.34	6.34	6.32	6.37	6.30	6.46	6.52
C	-	6.28	6.25	6.25	6.19	6.19	6.21	6.30	6.30	6.23	6.19	6.24
D	-	6.22	6.25	6.13	6.18	6.20	6.14	6.10	6.15	5.95	6.14	6.19
						NO_3 Rumen Fluid (mcg. NO_3/5 ml. rumen fluid)						
Treatment												
A	21.2	84.3	73.8	36.0	24.9	34.3	28.5	26.1	21.1	23.7	19.5	35.5
B	14.8	45.5	41.9	37.3	31.1	30.2	25.8	24.0	20.1	21.6	21.2	19.5
C	18.7	27.0	23.9	28.1	32.4	21.3	18.9	22.3	17.8	17.7	17.1	16.5
D	22.7	77.2	38.4	39.1	36.3	27.8	27.9	20.5	20.6	16.8	18.1	20.6
						NH_3-N Rumen Fluid (mg. NH_3-N/100 ml. rumen fluid)						
Treatment												
A	6.0	13.9	16.3	14.7	14.6	11.8	10.0	6.8	4.6	3.3	3.1	3.1
B	3.9	13.0	13.9	12.8	12.4	9.8	9.1	5.6	3.0	2.2	2.2	2.0
C	5.2	11.7	11.7	11.1	10.7	11.0	9.7	7.6	5.4	3.9	3.2	3.0
D	5.4	14.7	15.4	13.4	12.7	12.4	9.7	6.4	4.7	3.1	3.2	2.8
						CO_2 Rumen Fluid (m Mol./L. rumen fluid)						
Treatment												
A	41.4	47.6	51.5	49.0	48.1	49.7	44.2	42.1	43.1	41.4	43.4	42.0
B	51.6	56.7	57.6	52.8	51.3	49.3	49.8	48.8	49.6	50.2	51.5	49.8
C	32.4	39.4	38.7	38.6	36.1	35.3	35.0	36.2	33.1	34.4	34.4	34.7
D	41.6	47.2	46.2	42.0	40.9	45.0	39.6	37.3	35.5	35.9	36.8	36.5

[a] The steers had a 14-day preliminary period of eating the millet. The zero time sample was taken prior to feeding on the 15th day. The feeding was limited to one hour. The one-hour and subsequent samples were timed from when the feed was given to the steers.

[b] Average value of observations obtained for 4 steers in Latin Square.

as determined by statistical analysis of the slopes when rumen nitrate concentration was plotted against time after feeding (Table 21). A possible explanation for finding no differences in the rate of nitrate disappearance from the rumen due to molybdenum is that the control forage, which had not been fertilized with molybdenum, contained over 0.5 ppm of the element. This low level of molybdenum was apparently sufficient for the rumen microorganisms to build an effective nitrate reducing system. Tillman et al. (1965) found that the addition of 1 ppm of molybdenum to a purified diet provided for a faster rate of nitrate disappearance from the rumen than did a diet to which no molybdenum had been added. Nine hours after feeding, these workers reported little difference in the amounts of nitrate in the rumen. The molybdenum content of the control ration was not reported.

The high molybdenum forages, Treatments C and D, were consumed in smaller ($P < .01$) amounts than were the forages from Treatments A and B (Table 13). Reduced feed intake is not generally considered a symptom of molybdenum toxicity (Ferguson et al., 1943; Underwood, 1962).

TABLE 13

AVERAGE DAILY INTAKE OF MILLET BY STEERS

Plot (Treatment)	Average Daily Intake (kg.)
A (−Cu, −Mo)	4.5
B (+Cu, −Mo)	4.8
C (−Cu, +Mo)	2.6[a]
D (+Cu, +Mo)	3.5

[a] Significantly less ($P < .01$).

However, Lesperance and Bohman (1961) reported that molybdenum added to alfalfa or grass hay and cottonseed meal rations at 100 ppm decreased consumption by heifers fed these rations from weaning to one year of age. Although the level of molybdenum in the forage fed to the steers in Trial 2 was only 3 to 4.3 ppm, it seemed to be responsible for a highly significant decrease in forage consumption. Intake data were not obtained in 1964 when relatively high levels of molybdenum were fed to sheep. However, since the sheep in all four treatment groups lost an average of between 1 and 2.7 kg. of weight during a 2-month feeding period, there was probably no great difference in consumption of the forage due to molybdenum content.

There was no treatment effect on ammonia concentration of rumen contents. There was a significant effect ($P < .05$) on rumen ammonia levels due to period (Table 22). Overall, the rumen ammonia levels increased from the first to the fourth period and coincided with the increased intake of the millet. Since the ammonia would come from the fermentation of digesta, more rumen ammonia would be present when the animals were consuming greater amounts of millet. The pH of the rumen contents was statistically examined separately for the periods between one and eight hours and between two and eight hours (Table 23). There were no differences detected in either case due to treatment, animal or period. The rumen fluid total carbon dioxide concentration in steers that received the molybdenum fertilized forages (Treatments C and D) was reduced ($P < .05$) as shown in Figure 2. However, when the rumen fluid carbon dioxide levels were adjusted to account for forage intake, that is m Mol. total carbon dioxide per liter of rumen fluid per kg. of millet

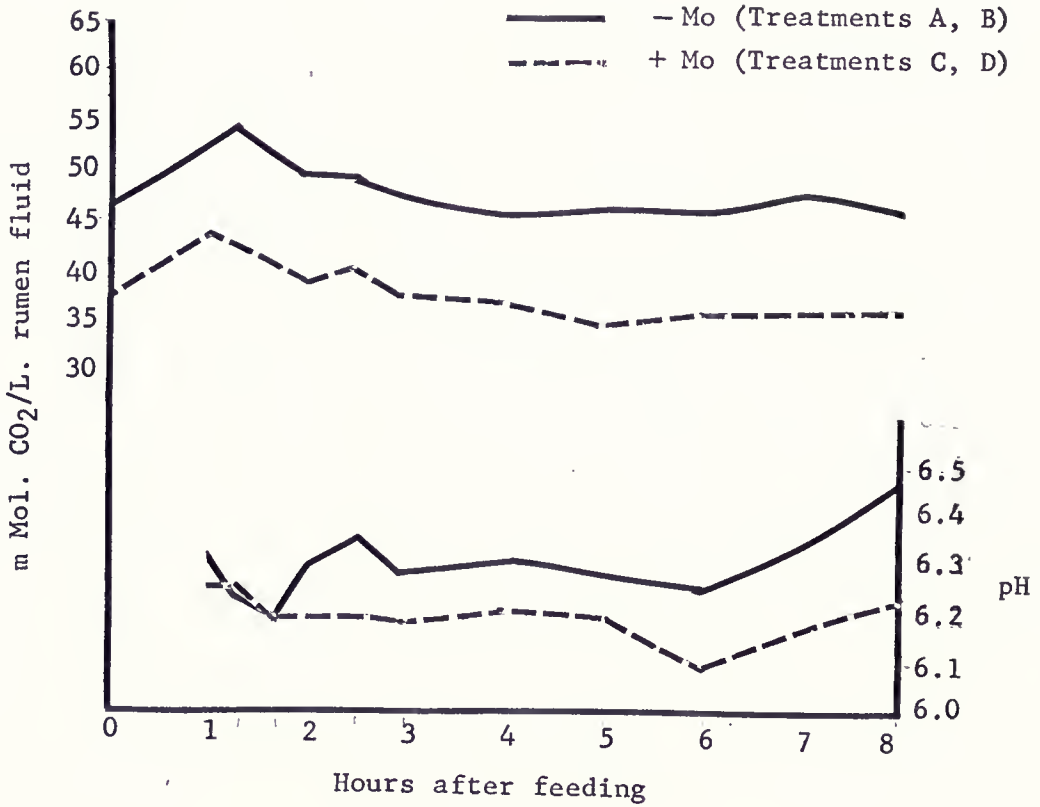

Figure 2. Summary of Effect of Molybdenum Forage Treatments on Concentrations of Carbon Dioxide and pH of Rumen Fluid.

consumed, the differences between the molybdenum treatments disappeared (Table 24).

Correlation coefficients were calculated for rumen pH against carbon dioxide levels. The coefficient for the treatments having no supplemental molybdenum (A, B) was 0.66 which indicated that 44.6% of the variation in rumen carbon dioxide levels could be explained by variations in pH. The coefficient for the molybdenum treatments (C, D) was 0.87 which means that 76.3% of the variation in rumen carbon dioxide levels was due to pH. Although the treatments did not significantly affect pH, there was a trend to higher pH values with higher carbon dioxide levels. This may have been caused by the increased levels of alkaline salts present with the higher levels of carbon dioxide.

There were no significant differences due to treatment in the total volatile fatty acid concentration in the rumen fluid (Table 25). The volatile fatty acid concentrations found in the rumen are shown in Table 26. Dobson (1961) said that about 1 molecule of bicarbonate appeared in the rumen for every 2 molecules of fatty acid absorbed. There was more of the low molybdenum forage consumed and presumably more fatty acids produced, yet there was no difference in the amount of fatty acids found in the rumen. If the presumed extra production of fatty acids was absorbed, more bicarbonate would be found in the rumen contents. This would explain the increased carbon dioxide concentration observed when the steers consumed the low molybdenum forage. Since the production of fatty acids is probably a function of intake, when the total carbon dioxide concentration in the rumen was put on the basis of units of forage intake, the difference between treatments disappeared.

Increased salivation with its high concentration of bicarbonate, accompanying the greater intake could also have contributed to the higher carbon dioxide levels in the rumen.

An increased ($P < .05$) acetic:butyric acids ratio was observed in the steers fed the molybdenum fertilized forages (Table 14). There was no difference in the acetic:propionic acids ratio or in the propionic:butyric acids ratio.

TABLE 14

AVERAGE RUMEN VOLATILE FATTY ACID RATIOS FROM STEERS, 1965

Treatment	A/P	A/B	P/B
-Mo (plots A & B)	3.54	8.82	2.50
+Mo (plots C & D)	3.66	10.44[a]	2.88

[a] Significantly higher ($P < .05$).

CHAPTER VII

THE EFFECT OF MOLYBDENUM AND COPPER ON NITRATE REDUCTION *IN VITRO*

The *in vivo* studies indicated that the nitrate reducing systems of the rumen were relatively insensitive to added molybdenum and copper. *In vitro* studies were conducted where greater control over the constituents of the fermenting media was possible. These studies were conducted to investigate (1) the effect of several levels of molybdenum on the rate of nitrate reduction, (2) the effect of tungsten on nitrate reduction and (3) the effect of added copper on nitrite reduction rates.

Experimental procedure

Fermentation flasks of 250 ml. capacity were placed in a water bath maintained at 39°C. for *in vitro* studies. Carbon dioxide gas was bubbled through the fermenting media by way of a glass tube opening at the bottom of the flask. Each flask contained 200 ml. of fermentation media with the composition shown in Table 15 (Quicke *et al.*, 1959).

The inoculum was prepared in the following manner: representative rumen samples were obtained from a mature Angus steer with a rumen fistula. The steer had been maintained for at least two years on Bermuda grass hay fed *ad libitum*, 900 gm. soybean meal daily, trace mineralized salt and vitamins A and D. Whole rumen contents were withdrawn from the fistula and strained through four layers of cheesecloth. The filtrate

TABLE 15

COMPOSITION OF FERMENTATION MEDIA

Constituent	Quantity/200 ml.
Urea	252 mg.
Na_2CO_3	400 mg.
$FeCl_3 \cdot 6H_2O$	8.8 mg.
$CaCl_2$	10.6 mg.
Na_2HPO_4	226 mg.
NaH_2PO_4	218 mg.
KCl	86 mg.
NaCl	86 mg.
$MgSO_4 \cdot 7H_2O$	23.3 mg.
Na_2SO_4	30 mg.
Valeric acid	50 mg.
Biotin	40 mcg.
PABA[a]	100 mcg.
Bermuda grass	4 gm.

[a] Para-aminobenzoic acid.

obtained was centrifuged at 250 times gravity for 3 minutes to sediment coarse particles and protozoa. Eighty ml. of the supernatent solution were used to inoculate each flask. Flasks were prepared in duplicate having the following composition:

Trial 1

1. 60 ml. H_2O (control)
2. Sodium molybdate to bring the concentration in the final volume to 40 ppm of molybdenum.
3. Potassium nitrate so as to have a final concentration of 0.003% nitrate ion.
4. Combination of #2 and #3 above.

Trial 2

5. 60 ml. H_2O (control)
6. Molybdenum at 2 ppm.
7. Molybdenum at 10 ppm.
8. Molybdenum at 50 ppm.
9. Molybdenum at 2 ppm plus 200 ppm of tungsten.
10. Molybdenum at 10 ppm plus 1000 ppm of tungsten.

Trial 3

11. 60 ml. H_2O (control)
12. Copper sulfate to final concentration of 2 ppm of copper.

There was a final volume of 200 ml. in each flask.

These flasks were allowed to ferment for a preliminary period of 4 hours at which time 15 ml. samples were withdrawn from each flask, mixed with ½ ml. concentrated hydrochloric acid and immediately frozen for nitrate analysis. All samples were handled in this manner except those obtained from Treatments 11 and 12. These samples were collected

and frozen immediately for nitrate and nitrite analysis. After the samples were obtained at 4 hours, 0.6 gm. of nitrate was added as a solution of potassium nitrate to each flask to bring the final concentration to approximately 0.03% of nitrate. The contents were sampled periodically for 7 to 8 hours.

The nitrate was added to Treatments 3 and 4 in an effort to stimulate the synthesis of a nitrate reducing system by the bacteria during the 4-hour preliminary period. Treatments 5 through 8 were an effort to determine the effect, if any, of varying levels of molybdenum on the rate of nitrate disappearance. Treatments 9 and 10 were efforts to determine the effect of tungsten, a molybdenum antagonist (Ramakrishna Karup and Vaidyanathan, 1963) on nitrate disappearance indirectly through molybdenum. Treatments 11 and 12 would indicate the effect that additional copper, at a level of 2 ppm, had on the disappearance rate of nitrite.

Results and discussion

The results from the first four treatments are shown graphically in Figure 3. Nitrate was added and it appeared that the molybdenum may have caused an increased rate of nitrate disappearance during the first hour of fermentation but this advantage was not apparent during the period from 2 to 8 hours. Preincubation with nitrate (Treatments 3 and 4) had no consistent effect on nitrate disappearance. Figure 4 shows the nitrate levels from Treatments 5, 6, 7 and 8. There was no increase in nitrate disappearance due to molybdenum. The higher the molybdenum levels the more nitrate was present at the end of 8 hours of fermentation. As can be seen from Figures 5 and 6, tungsten had no effect on nitrate reduction in these fermentation flasks.

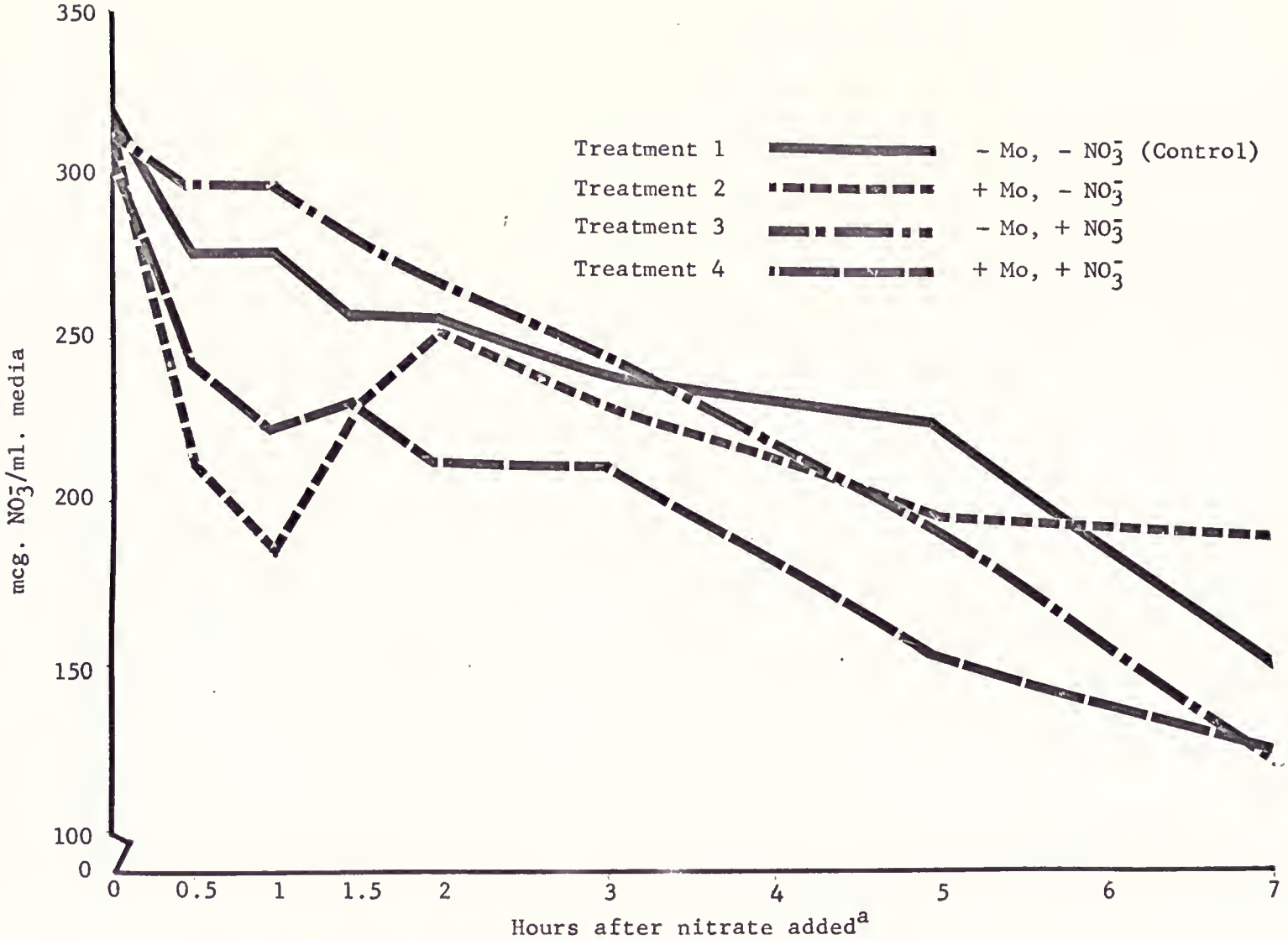

Figure 3. In vitro Nitrate Levels With and Without Nitrate and Molybdenum.

[a] Treatments 3 and 4 had 0.003% nitrate during a 4 hour incubation period with and without molybdenum. Nitrate was added to a final concentration of 0.03% to all treatment flasks at zero time.

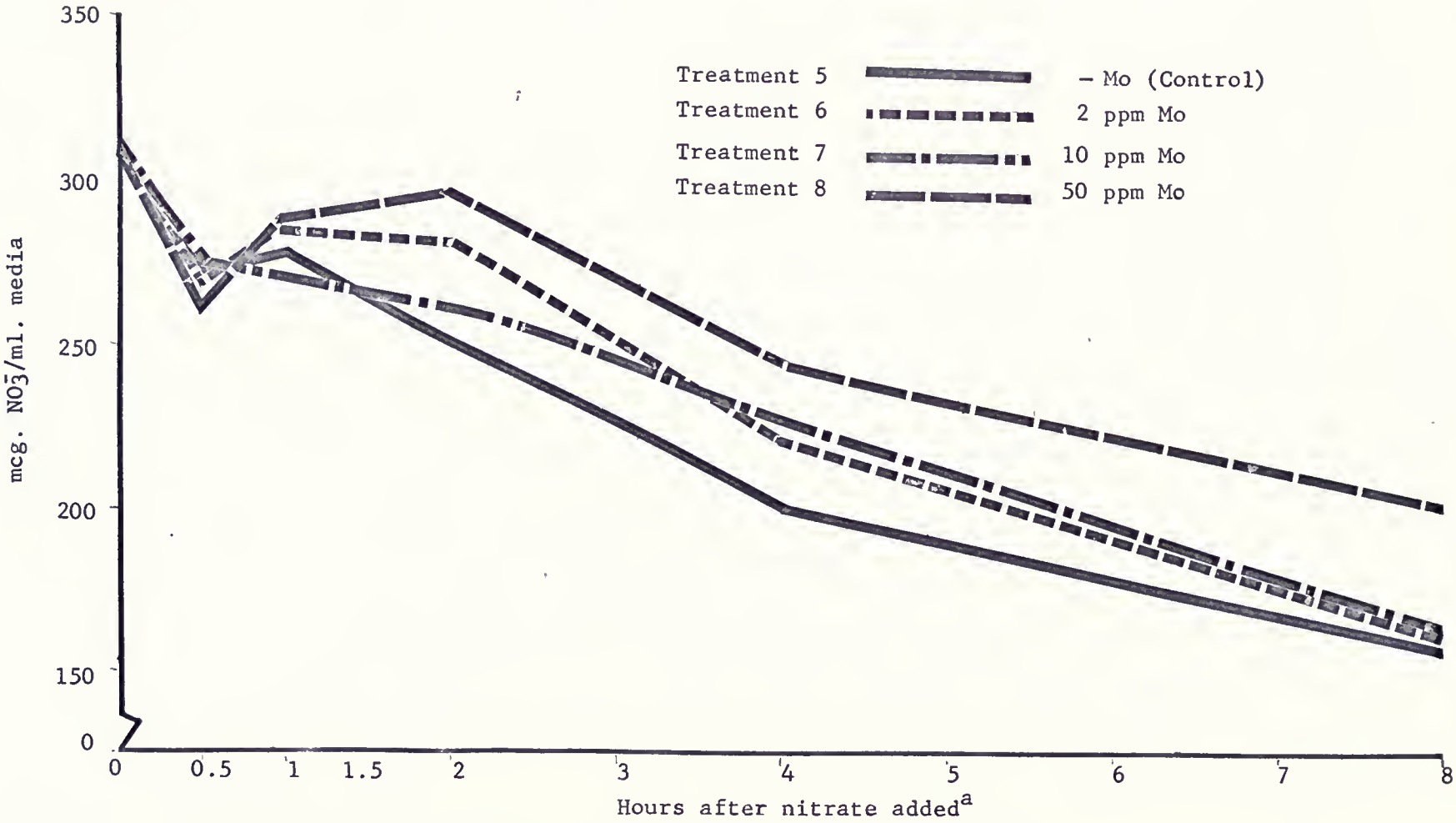

Figure 4. In vitro Nitrate Disappearance With Varying Amounts of Molybdenum.

[a] Samples were incubated 4 hours prior to addition of nitrate.

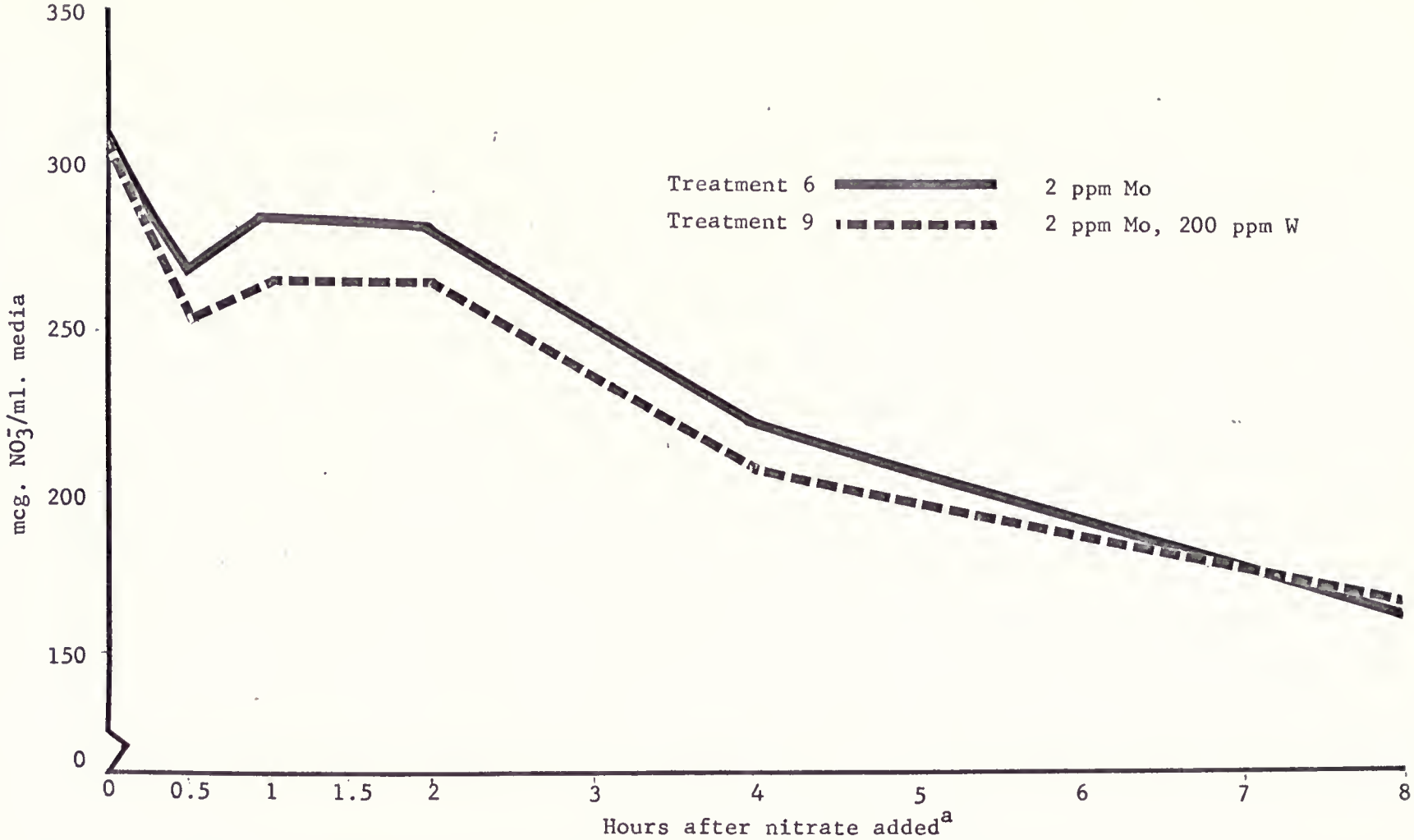

Figure 5. *In vitro* Nitrate Levels With Molybdenum and With and Without Tungsten.

[a] Samples were incubated 4 hours prior to addition of nitrate.

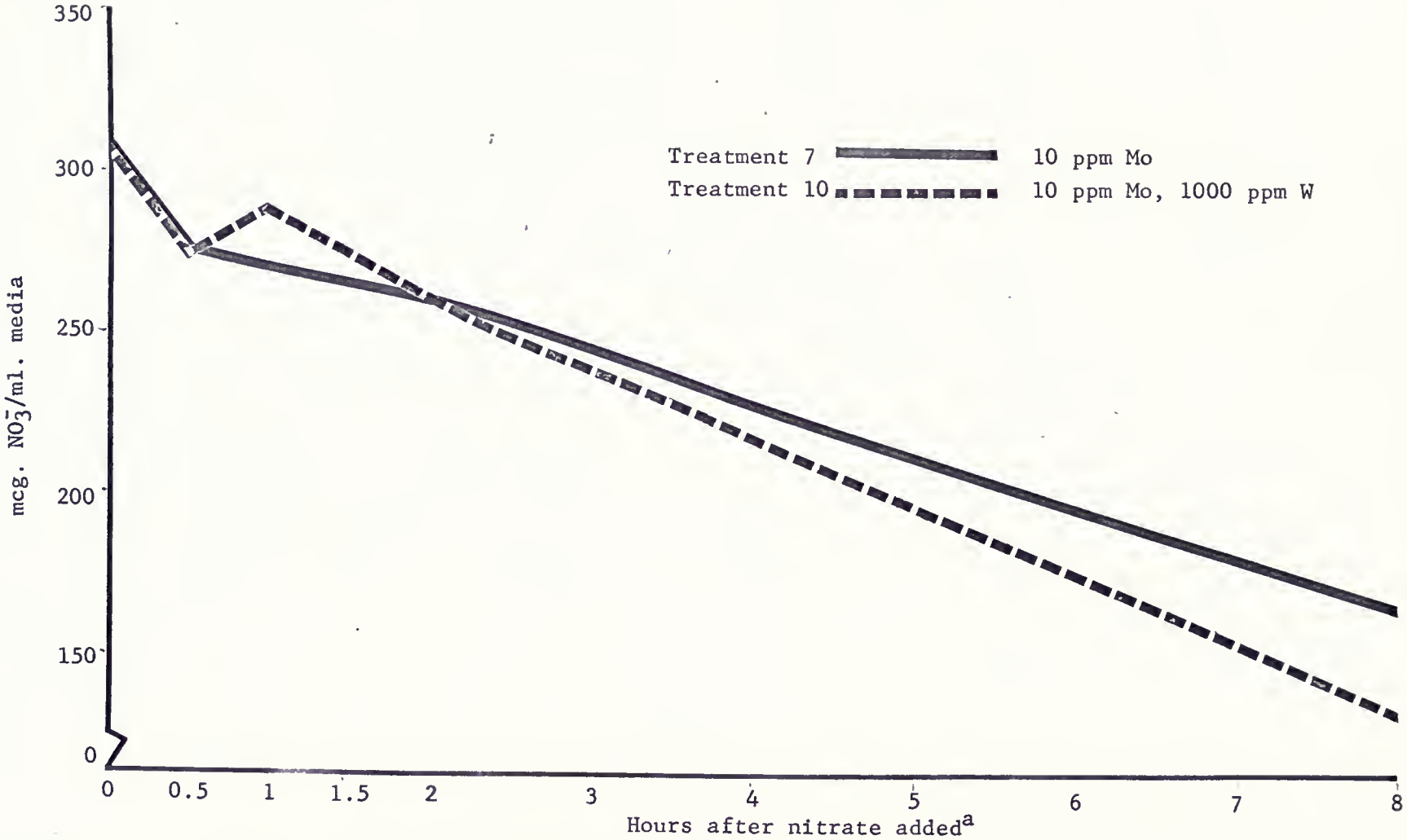

Figure 6. In vitro Nitrate Levels With Molybdenum and With and Without Tungsten.

[a] Samples were incubated 4 hours prior to addition of nitrate.

Figures 7 and 8 show the levels of nitrate and nitrite from Treatments 11 and 12 respectively. Copper at 2 ppm had no apparent effect on the rate of nitrate and nitrite disappearance. As the nitrate disappeared the nitrite increased. However, the nitrate decreased about 90 mcg. per ml. in 8 hours while the nitrite increased approximately 30 mcg. per ml. in the same period. This indicated that the nitrite was an intermediate compound in the nitrate to ammonia reduction chain.

A possible explanation for these results is that extremely low levels of molybdenum and copper are required for the nitrate and nitrite enzyme reducing systems. These very low levels of molybdenum and copper could have been carried into the system with the bacteria, as a contaminant in the media or in the Bermuda grass hay. This suggests that the bacteria should be washed several times to rid them of all excess molybdenum and copper before inoculating the media.

Generally, the *in vitro* studies support the *in vivo* work in that the nitrate reducing system seems to be indifferent to high levels of molybdenum and copper. Tungsten, in amounts 100 times those of molybdenum, had no antagonistic effect on molybdenum as determined by the rate of nitrate reduction.

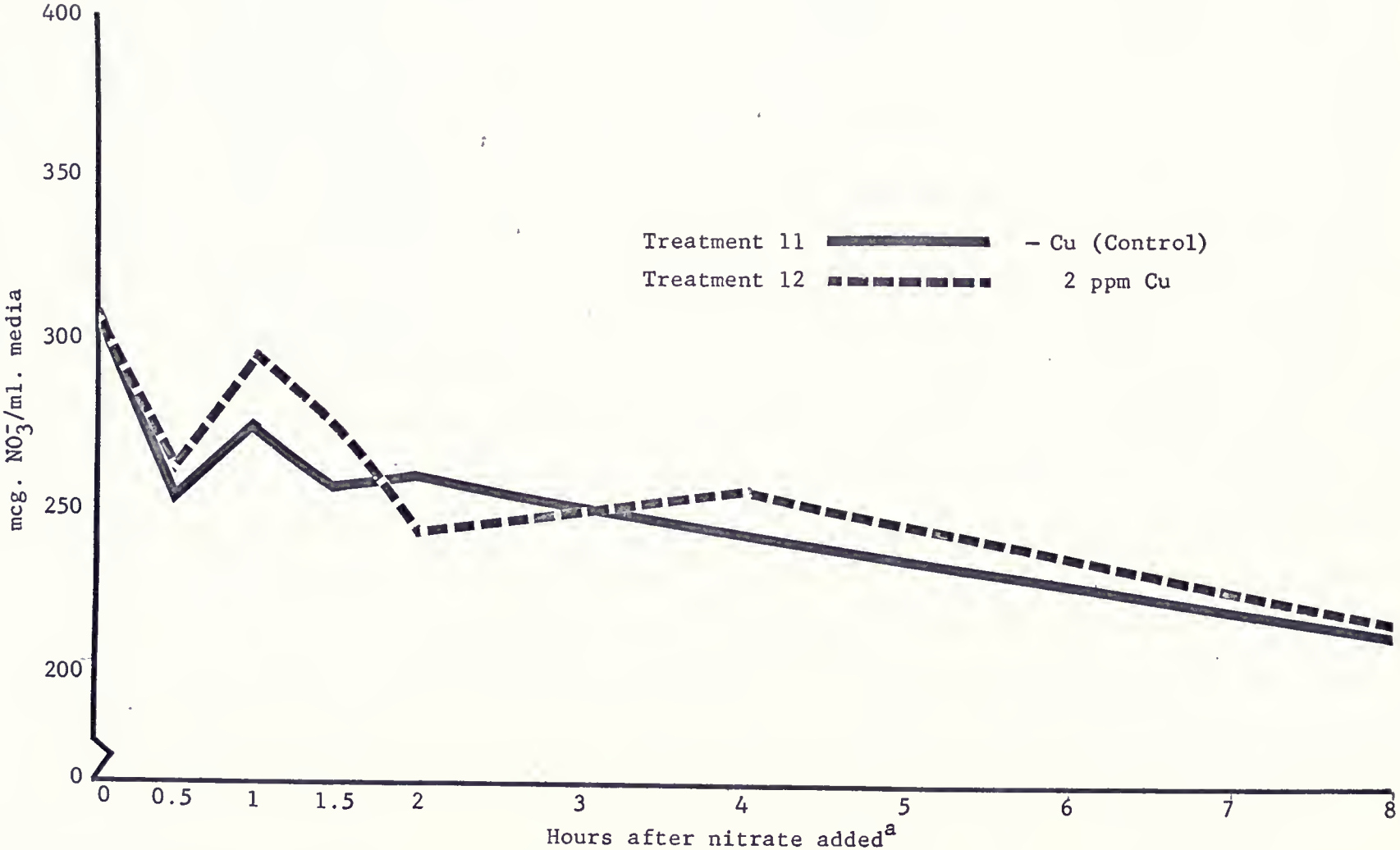

Figure 7. In vitro Nitrate Levels With and Without Copper.

[a] Samples were incubated 4 hours prior to addition of nitrate.

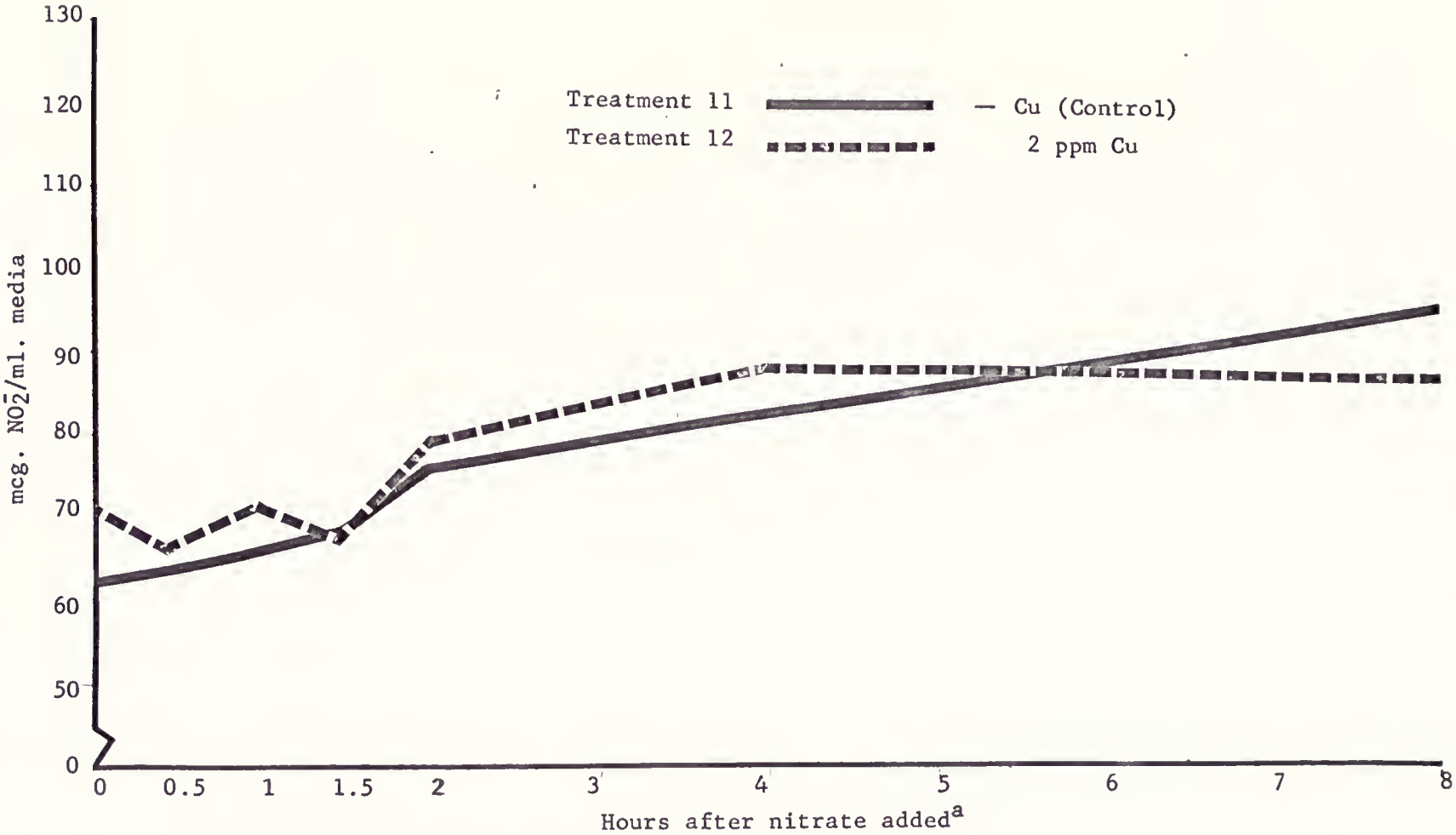

Figure 8. *In vitro* Nitrite Levels With and Without Copper.

[a] Samples were incubated 4 hours prior to addition of nitrate.

CHAPTER VIII

GENERAL DISCUSSION

The nitrate ion occupies a position of primary importance in the normal metabolism of higher plants. However, nitrate may accumulate within plants to very high levels with results disastrous to animals consuming these plants or to animals and humans exposed to the gaseous decomposition products. Acute poisoning has occurred in livestock and many cases of chronic poisoning have been reported. "Silo fillers disease" is a condition caused by inhalation of gases coming from silos recently filled with high-nitrate forage. Most of the early research concerned with nitrate toxicity was stimulated by sporadic but heavy losses of livestock. This early research was mainly in the form of surveys of the nitrate content of plants and reports of responses of animals when they were dosed with nitrate or nitrite salts. Recently more physiological and biochemical aspects have been investigated.

Molybdenum toxicity has been considered a problem involving copper and the sulfate ion with the molybdenum somehow making the copper unavailable to the tissues. Copper supplementation overcame the toxicity. Sulfate will decrease the molybdenum in the blood and tissues and increase its excretion in the urine. Under conditions of high molybdenum and high sulfate intake, the blood copper will increase rapidly and copper deficiency symptoms will be apparent (Dick, 1956). Skipper (1951) fed

young dairy bull calves 100 and 200 ppm of molybdenum per day for five months and Cox et al. (1960) fed up to 400 ppm of molybdenum to bull calves for three to six months in the feedlot without any toxic effects. These experiments suggested that some factor or factors may be present in pasture that were not present in the hay and concentrate fed these animals and that the toxicity was not due to high molybdenum levels per se.

The present investigation concerned the possibility that high levels of nitrate in forages were involved in the molybdenum-copper relationship. About the time these experiments were culminated, Tillman et al. (1965) at Oklahoma reported that a purified diet to which 1 ppm of molybdenum had been added, reduced nitrate at a faster rate than the same purified diet to which no molybdenum had been added. The molybdenum content of the control ration was not reported.

The present research indicated that rumen bacteria reduced nitrate at a maximum rate with 0.6 ppm of molybdenum. The minimum molybdenum requirement cannot be more accurately estimated from the results of this study. The purified diets used by the Oklahoma workers may be presumed to have contained less than 0.6 ppm of molybdenum unless the molybdenum used in their diets was less available to the rumen bacteria than was the molybdenum in the millet fed in the present experiments. The lack of sensitivity to molybdenum by cattle in the studies of Skipper (1951) and Cox et al. (1960) suggested that inorganic supplemental molybdenum did not act in the rumen like molybdenum naturally occurring in forage. Recent data published by Cook et al. (1966) indicated that there was very little difference in availability of the two types of molybdenum.

The nitrate reducing enzyme system did not respond to added molybdenum when rumen contents were incubated *in vitro*. Neither did the activity of the enzyme system decrease when tungsten was added. The failure of tungsten to interfere with molybdenum by replacing the latter in nitrate reductase did not concur with reports that have established tungsten as a molybdenum antagonist (Higgins *et al.*, 1956; Ramakrishna Karup and Vaidyanathan, 1963). In the present study it is possible that the nitrate reductase was present in the bacterial cell and was not synthesized to any appreciable extent during the four-hour preliminary fermentation period. This prior formation of nitrate reductase may have prevented the tungsten from effectively competing with molybdenum for incorporation into the enzyme. Prior formation of the enzyme would also explain the lack of effect on the rate of nitrate reduction when molybdenum was added to the *in vitro* system in amounts as high as 50 ppm. However, if nitrate reductase is an induced enzyme in rumen bacteria as it is in higher plants (Tang and Wu, 1959; Hewitt and Afridi, 1959), and *E. coli* and *Neurospora* (Nicholas, 1959), it is difficult to explain why the enzyme should have been present in maximal amounts before the addition of nitrate.

The forages high in molybdenum decreased the total carbon dioxide and increased the acetic:butyric acid ratios in the rumen contents but the relationship of these observations to the nitrate toxicity problem is not apparent.

The present research demonstrated that a nitrate drench compared to an equal amount of nitrate mixed in the feed produced more drastic effects on the same sheep consuming identical diets under a similar system of management.

The difficulty of growing experimental forages containing high levels of nitrate has been reported (Davison et al., 1965). The nitrate content of the millet grown for experimental purposes in 1964 and 1965 was much lower than the nitrate content of the millet grown at the Dairy Research Unit for routine feeding of the dairy herd. In future attempts to raise high nitrate forage, the author would recommend using much higher levels of nitrogen fertilizer with a minimum amount of irrigation.

This work suggests that the activity of nitrate reductase should be determined on rumen contents from cattle or sheep grazing forages containing different levels of nitrate, copper and molybdenum. Such a study would demonstrate how nitrate reductase activity was influenced by these three factors.

It would also be of interest to find the exact molybdenum requirement of cattle for maximum levels of nitrate reductase. This might be done using purified diets such as those used by Sheriha et al. (1962). In vitro studies using washed bacteria would also be of use in this type of determination.

The present study has demonstrated that there is little likelihood that a deficiency or an excess of molybdenum would be a practical factor in nitrate toxicity insofar as nitrate reduction to nitrite in the rumen is concerned. However, the present study did not demonstrate the importance of copper in nitrate toxicity. If the copper were too low or unavailable for the normal activity of nitrite reductase, a lack of copper would be of great significance as nitrite might accumulate to toxic levels. It may be that this accounts for the observed benefit of inorganic copper supplementation in salt mixtures when cattle graze pastures

which contain apparently adequate levels of copper. The author believes that further work relating to nitrate toxicity should be directed more toward the role of copper than that of molybdenum.

CHAPTER IX

SUMMARY

Molybdenum has been established as a component of nitrate reductase and copper functions in nitrite reductase activity. Since molybdenum toxicity symptoms can be alleviated with copper supplementation, it was considered that nitrate toxicity may have contributed to the molybdenosis symptoms. The purpose of this investigation was to determine if different levels of molybdenum and copper in forages were practical factors in the rate of nitrate disappearance from the rumen.

Millet was raised during 1964 and 1965 with high levels of nitrogen fertilization with and without supplemental molybdenum and copper. Sodium molybdate added at the rate of 0.9 kg. per acre increased the molybdenum content of forage from an average of 0.8 ppm in the control forage to 4 to 27 ppm. Copper sulfate applied at the rate of 11.35 kg. per acre resulted in copper levels of 10 to 11 ppm, except in a few samples which had surface contamination. Copper values on the unsupplemented forage ranged from 4 to 13 ppm.

Levels of forage molybdenum ranging from 0.6 ppm to 27 ppm had no consistent effect on the rate of nitrate reduction in the rumen of sheep and cattle, indicating that rumen microorganisms have an effective nitrate reducing enzyme system with as little as 0.6 ppm molybdenum. The high levels of molybdenum in the forage significantly decreased consumption

of the millet. There were increased acetic:butyric acid ratios and decreased carbon dioxide levels in the rumen contents of the steers fed the high molydenum millet. The treatments had no significant effect on pH or ammonia concentration in rumen fluid. There were also no treatment differences in the ammonia or methemoglobin levels in the blood.

Since additional molybdenum and copper in forage had no consistent effect on the rate of nitrate disappearance from the rumen, it can be concluded that the rumen nitrate reducing system does not depend on high levels of these two elements to function.

In vitro studies indicated that added molybdenum, tungsten or copper had no consistent effect on the rate of nitrate or nitrite reduction. These studies supported the in vivo studies in that the nitrate reducing system seemed to be relatively insensitive to additions of copper and molybdenum.

Sheep that consumed a purified diet were drenched with 16.8 gm. nitrate daily and the levels of methemoglobin were determined for as long as 15 hours after drenching. Although the sheep had consumed 16.8 gm. nitrate per day for two months preceding the drenching with no ill effects, when they were drenched with nitrate, methemoglobin rose to high levels and some of the sheep succumbed. The peak levels of methemoglobin generally rose from the first to the fifth day which indicated that there was a gradual loss of efficiency of the nitrate reducing system.

Water samples obtained from wells and rivers in Central Florida were analyzed for nitrate, nitrite and chloride content. The sample from one of the wells at Belle Glade and the Hillsborough River sample were

relatively high in nitrates but the rest of the samples contained only low levels of nitrate. Two samples from Belle Glade contained relatively high levels of nitrite which would have been potentially dangerous if used as a source of drinking water for livestock. Chloride levels were quite high in a few cases but most of the samples were low in chloride.

APPENDIX

TABLE 16

SUMMARY OF ANALYSIS OF VARIANCE OF SLOPES OF AMMONIA CONCENTRATION IN RUMEN OF SHEEP, 1964

Average Slopes				
Treatment A	Treatment B		Treatment C	Treatment D
-1.0308	2.7990		3.3888	-0.6644
Source	d.f.	M.S.	F	Sig.
Among Treatments	3	1.75	1.79	NS
A,B vs C,D	1	5.18	5.30	NS
Residual	2	0.08	0.04	NS
Error	8	0.98		
Total	11			

TABLE 17

INDIVIDUAL VALUES OF THE pH OF RUMEN FLUID FROM STEERS, 1965

Time[a]	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 77		
1	6.25	6.35	6.15	6.15
1:33	6.25	6.30	6.10	6.45
1:67	6.15	6.20	6.00	-
2	6.15	6.35	5.90	6.45
2:50	6.25	-	6.00	6.35
3	6.35	6.30	6.00	6.35
4	6.40	6.20	5.95	6.30
5	6.20	6.20	-	6.30
6	6.25	6.05	-	6.20
7	6.00	6.15	5.90	6.25
8	6.20	6.20	5.95	6.30
		Steer 107		
1	6.40	6.30	6.30	6.60
1:33	6.70	6.35	6.35	6.45
1:67	-	6.40	6.29	6.40
2	6.60	6.55	6.37	6.35
2.50	6.55	6.50	-	6.30
3	6.55	6.40	6.22	6.10
4	6.55	6.50	6.40	6.05
5	6.55	6.55	6.18	6.30
6	6.65	6.60	6.00	-
7	6.40	7.00	6.00	6.25
8	6.55	7.00	6.15	6.45

TABLE 17 (CONTINUED)

Time[a]	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 712		
1	6.60	6.40	6.30	6.08
1:33	6.40	6.10	6.30	6.05
1:67	6.45	6.15	6.40	5.98
2	6.35	6.30	6.25	5.85
2:50	6.30	6.32	6.35	-
3	-	6.32	6.40	6.10
4	6.40	6.25	6.65	6.00
5	-	6.35	6.55	5.98
6	-	6.25	6.50	5.75
7	6.90	6.40	6.60	5.90
8	7.15	6.45	6.60	5.90
		Steer 808		
1	5.82	6.40	6.35	6.05
1:33	5.80	6.25	-	6.05
1:67	5.85	6.25	6.30	6.00
2	5.90	6.20	6.25	6.05
2:50	-	6.20	6.22	5.95
3	5.80	-	6.22	6.00
4	5.85	6.35	6.20	6.05
5	5.80	-	6.15	6.05
6	5.70	-	6.20	5.90
7	5.60	6.30	6.25	6.15
8	5.75	6.45	6.25	6.10

[a] Hours after steers were fed.

TABLE 18

INDIVIDUAL VALUES OF THE NITRATE CONTENT OF RUMEN FLUID FROM STEERS, 1965

Time	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 77		
0	11[a]	-	30	15
1	210	29	45	46
1:33	138	34	45	34
1:67	20	40	48	31
2	21	30	58	27
2:50	26	-	53	26
3	20	16	43	15
4	15	16	49	12
5	12	16	34	11
6	14	10	49	10
7	15	14	44	9
8	15	6	53	9
		Steer 107		
0	15	0	-	30
1	68	23	33	225
1:33	78	34	32	65
1:67	24	42	46	65
2	26	28	52	85
2:50	28	27	-	48
3	15	20	22	68
4	15	25	22	40
5	13	16	28	49
6	9	24	5	35
7	13	13	11	46
8	10	12	0	60

TABLE 18 (CONTINUED)

Time	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 712		
0	38[a]	11	16	-
1	38	73	18	34
1:33	38	62	2	41
1:67	58	20	0	34
2	33	24	3	33
2:50	49	22	0	-
3	52	15	1	21
4	55	11	10	20
5	55	9	3	14
6	69	13	7	17
7	44	9	6	5
8	110	8	4	5
		Steer 808		
0	-	34	10	23
1	22	58	12	4
1:33	42	38	17	14
1:67	43	48	19	27
2	20	43	17	0
2:50	-	42	12	10
3	27	53	10	8
4	20	44	9	11
5	5	40	7	9
6	3	40	10	5
7	6	49	8	12
8	7	53	10	9

[a] mcg. NO_3^- per 5 ml. rumen fluid.

TABLE 19

INDIVIDUAL VALUES OF THE AMMONIA CONTENT OF RUMEN FLUID FROM STEERS, 1965

Time[a]	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 77		
0	5.07[b]	-	6.18	3.06
1	16.03	14.66	15.69	13.71
1:33	21.25	14.96	16.16	15.56
1:67	18.19	15.65	14.41	14.38
2	20.40	15.55	14.90	13.67
2:50	14.47	-	15.36	11.63
3	12.03	12.82	13.60	8.58
4	7.94	10.09	10.35	4.44
5	4.62	5.65	7.59	1.84
6	2.94	3.36	4.50	1.20
7	2.94	3.31	3.26	1.27
8	2.77	3.43	2.79	1.10
		Steer 107		
0	6.57	5.21	-	6.25
1	17.48	14.81	10.82	25.49
1:33	19.58	17.12	10.55	27.27
1:67	17.96	15.25	10.19	23.38
2	16.97	14.52	10.49	19.60
2:50	14.25	12.24	-	17.18
3	11.31	8.98	8.96	13.84
4	7.07	4.93	8.28	8.09
5	4.36	2.25	6.81	4.96
6	2.27	2.82	5.26	2.95
7	2.54	2.18	4.54	3.72
8	2.80	1.97	4.50	3.43

TABLE 19 (CONTINUED)

Time[a]	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 712		
0	6.20[b]	0.81	4.69	-
1	9.57	9.41	9.84	7.88
1:33	11.28	9.88	9.62	7.93
1:67	9.64	8.10	10.07	8.53
2	8.04	7.98	7.80	8.80
2:50	6.62	7.05	8.53	-
3	5.17	6.18	8.50	8.61
4	3.34	2.78	5.66	7.54
5	2.22	1.36	3.18	7.20
6	1.51	1.06	3.14	4.72
7	1.75	0.97	2.46	4.52
8	2.19	0.86	2.64	3.32
		Steer 808		
0	-	5.55	4.69	6.93
1	12.16	12.91	10.50	11.70
1:33	13.00	13.41	10.28	11.01
1:67	12.97	12.17	9.76	7.42
2	13.13	11.60	9.55	8.78
2:50	-	10.12	9.01	8.33
3	11.63	8.35	7.74	7.81
4	8.76	4.38	5.90	5.67
5	7.24	2.41	4.22	4.65
6	6.25	1.45	2.56	3.57
7	5.31	2.36	2.62	3.19
8	4.68	1.73	2.17	3.28

[a] Hours after steers were fed.
[b] Mg. NH_3-N per 100 ml. rumen fluid.

TABLE 20

INDIVIDUAL VALUES OF THE CARBON DIOXIDE CONTENT OF RUMEN FLUID FROM STEERS, 1965

Time	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 77		
0	40.70[a]	-	26.59	51.18
1	47.22	58.45	34.84	60.19
1:33	56.43	55.53	33.50	64.22
1:67	49.20	54.56	30.15	59.79
2	44.47	52.88	27.73	60.21
2:50	41.41	-	27.92	57.57
3	43.00	55.32	30.13	52.14
4	44.21	53.68	28.50	46.42
5	43.30	57.56	26.91	46.12
6	36.94	52.12	24.94	46.61
7	40.38	52.17	24.48	49.44
8	41.37	54.73	24.23	47.23
		Steer 107		
0	53.93	57.84	-	46.48
1	65.78	60.48	42.38	54.12
1:33	69.78	73.03	36.44	59.51
1:67	69.88	66.37	39.31	51.09
2	70.41	61.94	37.64	45.81
2:50	62.22	57.90	-	47.89
3	64.44	56.09	38.02	46.43
4	59.66	57.23	37.42	43.23
5	57.10	52.33	38.90	38.61
6	55.05	54.00	38.58	41.52
7	56.11	53.79	37.71	44.51
8	56.54	45.21	36.70	46.14

TABLE 20 (CONTINUED)

Time	Treatment A	Treatment B	Treatment C	Treatment D
		Steer 712		
0	29.71[a]	52.82	31.10	-
1	45.92	57.47	35.68	-
1:33	49.94	56.06	43.68	24.79
1:67	47.70	48.68	42.39	25.12
2	47.33	49.30	39.62	26.30
2:50	45.40	49.48	37.71	-
3	41.63	47.22	35.50	30.00
4	37.49	47.88	41.03	27.43
5	40.56	48.15	31.42	27.71
6	42.39	49.36	37.28	24.83
7	47.85	52.80	36.48	24.29
8	39.04	55.40	38.01	22.86
		Steer 808		
0	-	44.03	39.45	27.24
1	31.38	50.52	44.50	34.02
1:33	29.86	45.65	41.13	36.31
1:67	29.05	41.72	42.58	32.12
2	30.02	40.97	39.26	31.39
2:50	-	40.61	40.38	29.42
3	27.70	40.59	36.38	29.68
4	27.21	36.47	37.68	32.26
5	31.55	40.44	35.10	29.55
6	31.22	45.50	36.92	30.76
7	29.42	47.09	38.96	28.76
8	31.03	43.72	40.04	29.63

[a] Milli moles total CO_2 per L. rumen fluid.

TABLE 21

SUMMARY OF ANALYSIS OF VARIANCE OF NITRATE FOUND IN RUMEN OF STEERS, 1965

Nitrate Found in Rumen at 4 Hours

Sources	d.f.	M.S.	F	Sig.
Animals	3	14	0.04	NS
Treatment	3	23	0.07	NS
Period	3	1043	3.17	NS
Error	6	329		
Total	15			

Nitrate Found in Rumen at 4 Hours per gm. Nitrate Fed

Sources	d.f.	M.S.	F	Sig.
Animals	3	10	3.0	NS
Treatment	3	11	3.3	NS
Period	3	16	4.8	5%
Error	6	3.33		
Total	15			

Slope of Curve of Nitrate Concentration Against Time After Feeding

Sources	d.f.	M.S.	F	Sig.
Animals	3	17.4	1.01	NS
Treatment	3	30.6	1.77	NS
Period	3	0.2	0.01	NS
Error	6	17.2		
Total	15			

TABLE 22

AVERAGED RUMEN AMMONIA LEVELS BETWEEN ONE AND TWO HOURS AFTER FEEDING

Sources	d.f.	M.S.	F	Sig.
Animals	3	9.33	0.95	NS
Treatment	3	9.53	0.97	NS
Period	3	57.20	5.83	5%
Error	6	9.80		
Total	15			

TABLE 23

SUMMARY OF ANALYSIS OF VARIANCE OF pH FOUND IN RUMEN FLUID OF STEERS, 1965

Average pH of Rumen Contents Between 1-8 Hours After Feeding

Sources	d.f.	M.S.	F	Sig.
Animals	3	0.083	2.52	NS
Treatment	3	0.027	0.82	NS
Period	3	0.077	2.33	NS
Error	6	0.033		
Total	15			

Average pH of Rumen Contents Between 2-8 Hours After Feeding

Sources	d.f.	M.S.	F	Sig.
Animals	3	0.09	2.16	NS
Treatment	3	0.043	1.00	NS
Period	3	0.10	2.32	NS
Error	6	0.042		
Total	15			

TABLE 24

SUMMARY OF ANALYSIS OF VARIANCE OF TOTAL CARBON DIOXIDE FOUND IN RUMEN FLUID OF STEERS, 1965

Total Carbon Dioxide (mMol. CO_2/liter rumen fluid) in Steers, 1965

Sources	d.f.	M.S.	F	Sig.
Animals	3	187.	5.23	5%
Treatment	3	191.	5.34	5%
AB vs CD	1	448.	12.51	5%
Residual	2	63.	1.76	NS
Period	3	149.	4.17	NS
Error	6	35.8		
Total	15			

Total Carbon Dioxide (mMol. CO_2/liter rumen fluid per kg. millet consumed) in Steers, 1965

Sources	d.f.	M.S.	F	Sig.
Animals	3	803.	4.0	NS
Treatment	3	414.	2.08	NS
AB vs CD	1	305.	1.54	NS
Residual	2	468	2.35	NS
Period	3	218.	1.10	NS
Error	6	199.		
Total	15			

TABLE 25

SUMMARY OF ANALYSIS OF VARIANCE OF TOTAL VOLATILE FATTY ACID CONCENTRATION AND RATIOS IN THE RUMEN FLUID OF STEERS, 1965

The Total Volatile Fatty Acid Concentration (m equiv./L. rumen fluid) in Steers

Sources	d.f.	M.S.	F	Sig.
Animals	3	464	2.91	NS
Treatment	3	355	2.23	NS
Period	3	248	1.55	NS
Error	6	159		
Total	15			

The Acetic:Propionic Acids Ratio in Steers

Sources	d.f.	M.S.	F	Sig.
Animals	3	1.54	25.67	1%
Treatment	3	0.07	1.08	NS
Period	3	0.01	0.15	NS
Error	6	0.065		
Total	15			

The Propionic:Butyric Acids Ratio in Steers

Sources	d.f.	M.S.	F	Sig.
Animals	3	0.56	0.187	NS
Treatment	3	0.28	0.094	NS
Period	3	0.10	0.033	NS
Error	6	2.99		
Total	15			

The Acetic:Butyric Acids Ratio in Steers

Sources	d.f.	M.S.	F	Sig.
Animals	3	3.14	2.91	NS
Treatment	3	6.42	5.94	5%
AB vs CD	1	10.59	9.80	5%
Residual	2	4.34	4.01	NS
Period	3	0.62	0.57	NS
Error	6	1.08		
Total	15			

TABLE 26

VOLATILE FATTY ACIDS FOUND IN THE RUMEN CONTENTS OF STEERS TWO HOURS AFTER CONSUMING HIGH NITRATE MILLET

Steer	Acid	Treatment A	Treatment B	Treatment C	Treatment D
77	Acetic	68.6[a]	48.8	55.8	64.9
	Propionic	19.4	13.6	16.3	17.4
	Butyric	7.7	6.6	6.9	6.0
107	Acetic	57.6	57.7	33.3	81.7
	Propionic	15.1	17.1	9.5	20.0
	Butyric	5.8	6.5	2.9	7.2
712	Acetic	48.2	40.0	46.6	44.7
	Propionic	14.9	12.5	13.4	14.6
	Butyric	4.4	6.2	4.0	3.9
808	Acetic	52.1	47.5	36.6	43.9
	Propionic	13.1	13.4	9.6	10.5
	Butyric	6.1	5.1	4.1	4.6

[a] Milliequivalents per liter.

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BIOGRAPHICAL SKETCH

David Thomas Buchman was born in Baltimore, Maryland, on November 17, 1933. He graduated from Catonsville High School in 1951. He then worked as a carpenter and in a machine shop until the spring of 1952 at which time he started to farm on the family dairy farms. Between June and December, 1957, he was a Private in the U. S. Army Reserve at Fort Jackson, South Carolina, and he entered the University of Maryland in February, 1958. He received the Bachelor of Science degree with honors in June, 1961, and the Master of Science degree in January, 1963, from the University of Maryland. He worked part-time at the University of Maryland dairy barns and as a Physical Science Aid at the USDA in Beltsville, Maryland, during his undergraduate years. At present he is a candidate for the degree Doctor of Philosophy in the Department of Animal Science, University of Florida.

He was married in June, 1956, to Maureen Elaine Michael of Shenandoah, Virginia. He is a member of the American Dairy Science Association, American Society of Animal Science, Alpha Zeta, Sigma Xi, Gamma Sigma Delta and Phi Kappa Phi.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April, 1966.

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