MOLYBDENUM IN RUMINANT NUTRITION

By

GIUMA M. SHERIHA

Bachelor of Science
Cairo University
Cairo, Egypt
1957

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1961

Submitted to the faculty of the Graduate School of the
Oklahoma State University of Agriculture and
Applied Science in partial fulfillment
of the requirements for the degree of
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Thesis Approved:

Allen D. Tillman
Thesis Advisor

Rollin H. Thayer

Hue Reuber

Robert D Morrison

George R. Waller

Dean of the Graduate School

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

Experiment I

Experiment II

Experiment III

LIST OF FIGURES

Experiment I

Experiment II

INTRODUCTION

Molybdenum is an essential element in animal and plant metabolism. Recent experimental results indicate that this element is concerned with nitrate reduction in plants and with purine metabolism in animals, thus is essential for the growth and well-being of higher forms of plant and animal life.

For many years, the main interest in molybdenum in animal nutrition concerned its toxic effect upon animals subsisting on forages containing high levels of this element. Such plants were found to exist in most of the countries in the world, thus much research effort has been expended in studying the toxic effects of this element. The results of such studies indicate that there is a close relationship between dietary levels of molybdenum, copper and sulfur in molybdenum toxicity cases.

Heavy nitrogen fertilization results in a great increase in the nitrate content of plants and there are indications that the vitamin A status of ruminants consuming such plants is decreased. As there are several intermediates in the metabolic pathway of reducing nitrates by ruminant animals to ammonia, it is possible that one of the intermediates could be concerned with the destruction of vitamin A in the diet or in the body.

The purposes of the studies reported herein were to study the role of molybdenum in ruminant nutrition. For convenience, the study is presented as three separate experiments as follows: Experiment I - Molybdenum

in nitrate utilization; Experiment II - The toxicity of hydroxylamine; and Experiment III - The interrelations of copper, sulfate sulfur and molybdenum in nutrition.

EXPERIMENT I

MOLYBDENUM IN NITRATE UTILIZATION

REVIEW OF LITERATURE

Utilization of Nitrate by Microorganisms

Nitrate. Verhoeven (1956) differentiated between three types of nitrate reduction as follows:

1. Assimilation, in which nitrate is reduced only for the building up of cell protein.
2. Incidental dissimilation, in which nitrate acts as a non-essential hydrogen acceptor.
3. True dissimilation, in which nitrate acts, at least under certain conditions, as the essential hydrogen acceptor which enables the organism to grow.

Sato (1956a) suggested that the two major functions of nitrate reduction are "nitrate assimilation" and "nitrate respiration." The purpose of nitrate assimilation is to utilize the products of nitrate reduction to build up the cellular nitrogeneous constituents. The nitrate respiration, on the other hand, is concerned with the energy supply to the cellular activities in anaerobiosis, and the nitrate is reduced as a hydrogen acceptor in energy-yielding processes. The reduction products are not needed in this case and are excreted in the form of nitrite, nitrous oxide, nitrogen, or ammonia. The ability to perform these two types of nitrate reductions, seems to be shared by different organisms and to a different extent. Thus, some organisms reduced nitrate almost solely for assimilative purposes, whereas in others nitrate respiration is

predominant over the assimilation.

Sato (1956b) also speculated that the nitrate assimilation should be attributed to the action of the molybdoflavoprotein enzymes and the nitrate respiration to that of the cytochrome mechanisms. Since the oxidation of cytochromes concomitant with the reduction of nitrate has so far been observed only in those organisms which are known to be able to perform the nitrate respiration and, secondly, all the organisms whose cytochromes are not affected by nitrate seem to lack the ability for nitrate respiration, but are able to utilize nitrate for assimilation, it appears plausible to assume that the cytochrome mechanisms function in nitrate respiration, which is so closely analogous to aerobic respiration. Some organisms may contain both mechanisms at the same time.

Taniguchi, Sato and Egami (1956) found, in cell-free extracts of E. coli, grown in a peptone broth-agar medium containing nitrate, a particulate system which reduced nitrate to nitrite only under anaerobic conditions and which reduced diphosphopyridine nucleotide, formate, or reduced methylene blue as electron donors and flavin nucleotides as electron carriers.

They propose that anaerobic nitrate reduction via flavin takes place through an electron-transport chain with some of the electron carriers in common with the DPNH-oxidase chain. Nitrate with terminal nitrate reductase can serve as the terminal respiratory acceptor in anaerobiosis, instead of oxygen plus terminal oxidase, which is the normal acceptor in aerobiosis. They called this type of nitrate reduction "nitrate respiration." The concept of "nitrate respiration" necessarily leads to the assumption that the formation of energy-rich phosphate compounds must be coupled to the anaerobic reduction of nitrate.

They pointed out also that nitrate respiration is not the only metabolic form of nitrate reduction E. coli is able to perform, because they found that the same cell suspension capable of carrying out nitrate respiration can also reduce nitrate, nitrite, and hydroxylamine to ammonia or probably even to cellular nitrogenous constituents when the suspension is sufficiently aerated and an adequate amount of respiratory substrate such as glucose is supplied. The enzyme systems responsible for these aerobic reductions must be quite different from nitrate reductase system functioning in the anaerobic process, since the latter is markedly inhibited by oxygen and would not play any role under aeration. The metabolic function of these aerobic reduction processes appears to be the assimilation of inorganic nitrogen compounds.

Nason (1958) stated that the essential feature of the metabolic interconversions of nitrate nitrogen is the oxidation-reduction change in the state of the nitrogen atom ranging between the extreme of plus five and minus three. Thus, nitrate assimilation, nitrate respiration, and denitrification (a form of nitrate respiration) are the result of the stepwise reduction of nitrate to the more negative oxidation states of nitrogen.

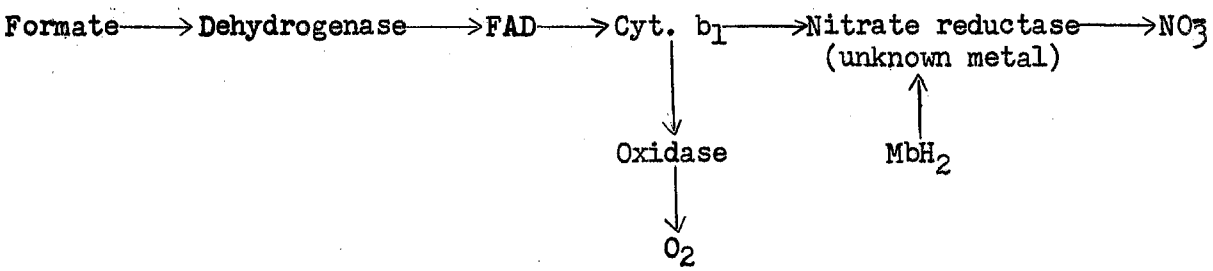
An important difference between nitrate respiration and nitrate assimilation is that the former is presumably coupled to high-energy phosphate esterification since nitrate can take the place of oxygen as a terminal electron acceptor. The nitrate reductase of nitrate assimilation is a molybdoflavoprotein, whereas the nitrate reductase of nitrate respiration appears to be a cytochrome system. The latter bears more structural resemblance to the established terminal oxidative phosphorylation system in animal mitochondria and very likely in bacterial preparations.

Takahashi and Taniguchi (1956) found that phosphorus turnover in resting cells of E. coli, which also showed nitrate respiration, was greatly enhanced by the addition of nitrate and formate under anaerobic conditions. When either nitrate or formate was omitted from the system, the rate of turnover significantly decreased. The results, though indirect, may suggest the occurence of coupled phosphorylation to the electron transport system between formic dehydrogenase and nitrate reductase.

Wainwright (1955) found that nitrate-reductase activity in E. coli strain 1431, was enhanced in anaerobiosis by vitamin K_3 and ferrous iron. The maximal activity of the enzyme was obtained when both vitamin K_3 and flavin nucleotides were added to the incubation mixture.

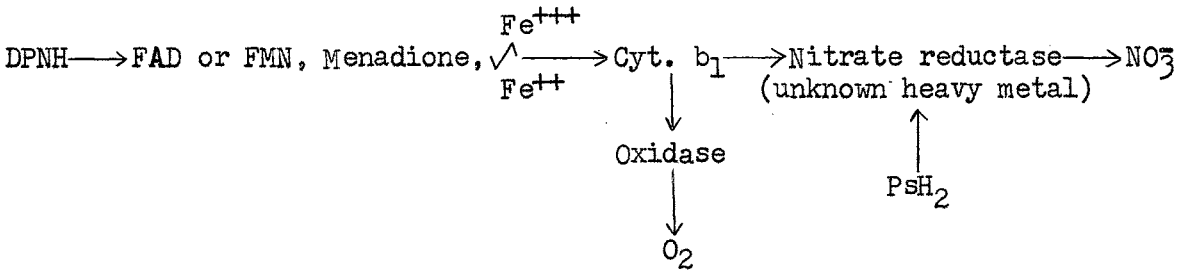
Lida et al. (1959) have shown that nitrate reductase, cytochrome b_1, formate and DPNH dehydrogenases, to be localized in the particulate fractions in the ultrasonic extract from E. coli, suggesting the residence of these four enzymes on a common particulate structure forming the particulate nitrate reductase system. In contrast with ultrasonic treatment, glycine treatment of E. coli cells yielded none of smaller particulate preparation, but the larger insoluble cellular fragments, on which nitrate reductase, cytochrome b_1 and formic dehydrogenase resided.

The whole sequence of electron transfer in the particulate nitrate reductase system can be summarized by the following scheme:



The enzymic electron transfer from reduced methylene blue to nitrate was insensitive towards 2-heptyl-4-hydroxyquinoline-N-oxide, indicating no participating of cytochrome b_1, in contrast with formate- or DPNH-nitrate reductase which was sensitive towards the inhibitor. Some heavy metal component was suggested to be tightly bound in nitrate reductase from the inhibitory action of cyanide and azide.

Itagaki and Taniguchi (1959) found that E. coli (yamagutchi strain) aerobically grown in the synthetic medium never assimilated nitrate. About 30 percent of the nitrate reductase activity (assayed by using reduced phenosafranine) of the whole cells was found in the soluble extract (107,000 g. supernatant). The soluble extract contained DPNH-nitrate reductase of nitrate respiration type which consisted of flavin, menadione, Fe^{++}, cytochrome b_1 as intermediary electron carriers and of sulfhydryl group.

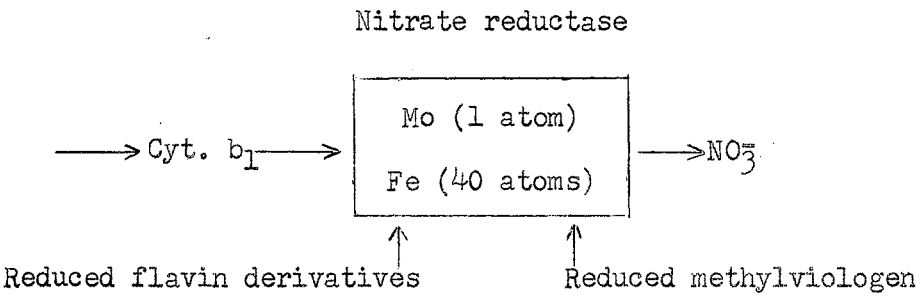


The cyanide and quinoline-N-oxide sensitive DPNH-oxidase probably involving cytochrome b_1 was also found in the soluble extract. In the soluble extract, Fe^{++} could enzymically donate its electron to nitrate probably via cytochrome b_1 with the comparative rate as from DPNH. Formate-nitrate reductase never demonstrated in the soluble extract but resided only in the particulate fraction. The purified enzyme (assayed by using reduced phenosafranine) had K_m of 7.5×10^{-5} M for enzyme-nitrate complex, pH optimum at 6.4 and was activated by citrate or

pyrophosphate.

Taniguchi and Itagaki (1960a) reported that a particulate nitrate reductase system which included cytochrome b_1 as an intermediary carrier from formate or DPNH to nitrate was prepared from E. coli cells grown anaerobically in the presence of nitrate. This system was found to possess remarkably high activity of nitrate reductase of the nitrate respiration type. The particulate preparation, devoid of activities suggestive of not only reduced pyridine nucleotide oxidases but also dehydrogenases except for formate was, as far as has been tested, functionally characterized by its highly active formate-nitrate reductase system. Nitrate reductase, the terminal enzyme of the particulate system, was solubilized by cold alkaline incubation after heat treatment and then purified to homogeneous state. The enzyme was shown to have a molecular weight of a million and to contain one atom of bound molybdenum and about 40 atoms of bound iron per molecule but no bound flavin. A difference spectrum (oxidized minus reduced) having a broad peak at 445 to 450 mμ disappeared rapidly on the addition of nitrate with simultaneous production of nitrite. The turnover number of the enzyme was estimated to be about 7.0×10^3 molecules per second, determined by the use of reduced methylviologen, a most effective electron donor. Examination of the physiological donors for the purified enzyme showed that reduced forms of pyridine nucleotides were never effective and flavin derivatives were but little effective. Data which suggested that reduced cytochrome b_1 would be more favorable donor for nitrate reductase were presented. The homogeneously purified enzyme seems to function as a terminal enzyme in a nitrate-respiring system in vivo and may be regarded as an anaerobic variant of terminal oxidases.

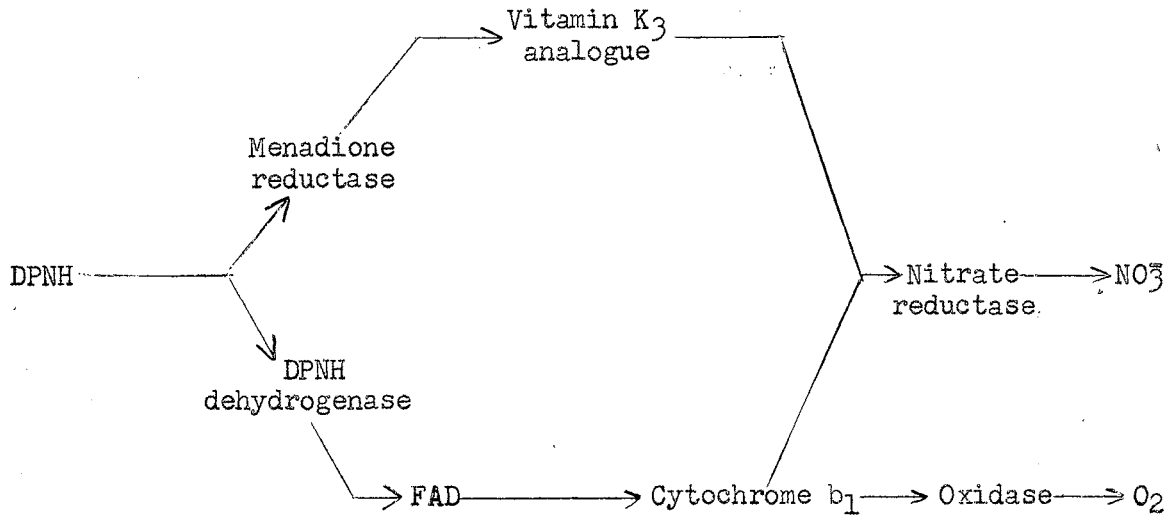
The electron-transferring sequences of this nitrate reductase system can be schematized as follows:



Heredia and Medina (1960) stated that a system which reduced nitrate to nitrite with di- or tri-phosphopyridine nucleotide as electron donor and vitamin K_3 as electron carrier, has been found in E. coli (strain 86).

The transfer of electrons from pyridine nucleotides to vitamin K_3 is an inducible enzyme which is associated with particulate matter. Nitrate reductase apparently involves some metal but not flavin, and had pH optimum at six to seven.

Anaerobically, in addition to this pathway, nitrate reduction can take place through a system, apparently flavin-dependent, presumably related to the reduced diphosphopyridine nucleotide oxidase. Only the identified steps are included in the following minimal scheme.

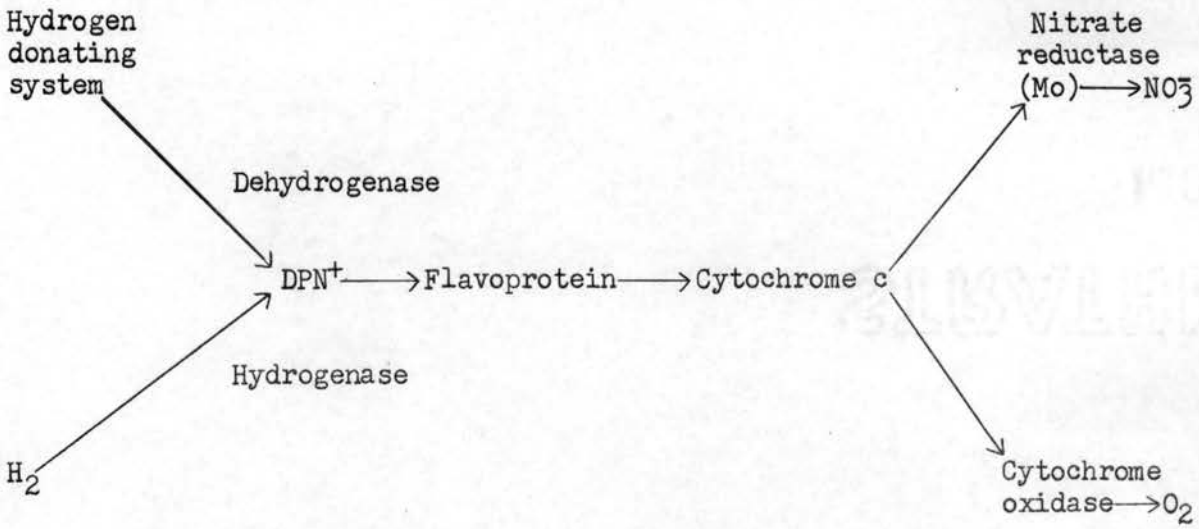


McNall and Atkinson (1956) reported that the E. coli strain Bn, which uses nitrate or nitrite as a source of nitrogen for growth, was isolated from a parental strain B which is strongly inhibited by nitrate or nitrite. Strain Bn reduced nitrate to ammonia at the expense of hydrogen, whereas the hydrogen uptake of the parental culture corresponds to reduction only to nitrite. An inhibitory level of nitric oxide, might, however be produced without an observable increase in total hydrogen uptake over that required for nitrite production. Strain Bn is much more tolerant to nitric oxide than is the parental type, apparently because the oxide is metabolized by the new strain. This ability of the strain Bn to prevent accumulation of oxide may account for the observed difference between strains in response to nitrate and nitrite.

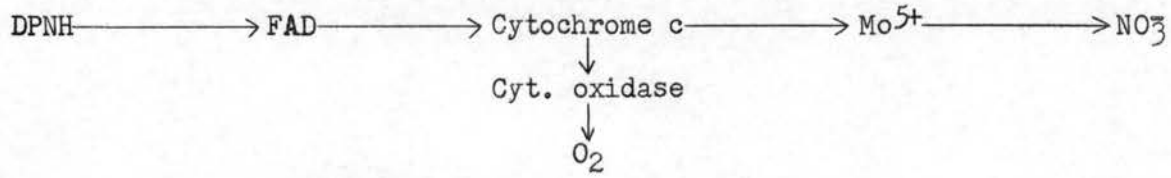
McNall and Atkinson (1957) found that the E. coli strain Bn, previously shown to grow with nitrate or nitrite as sole source of nitrogen, can similarly utilize hyponitrite, hydroxylamine, or nitrous oxide.

Fewson and Nicholas (1961a) found that the DPNH dependent nitrate reductase from Micrococcus denitrificans were active only in cells grown aerobically but increased 5-fold in those grown anaerobically. Nitrate reductase activity with DPNH or H_2 as the donor was depressed in bacteria deficient in either iron or molybdenum.

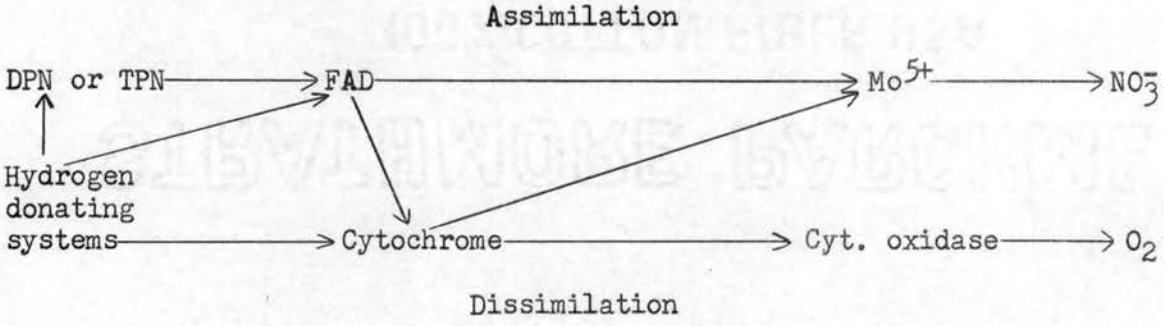
The reduced cytochrome c was oxidized immediately on the addition of nitrate. The sequence of electron transfer in M. denitrificans is probably:



Fewson and Nicholas (1961b) reported that nitrate reductase has been purified 115-fold from actively denitrifying cells of Pseudomonas aeruginosa. The enzyme is DPNH dependent and contains FAD, cytochrome c and molybdenum as functional components. Sulfhydryl groups are appeared to be involved in binding the FAD to the apoenzyme since p-chloromercuribenzoate competitively inhibited stimulation of the apoenzyme by FAD. Cells deficient in either molybdenum or iron showed a marked decrease in nitrate reductase activity. When Mo^{99} and Fe^{59} were incorporated into the culture medium both metals were concentrated in the purified enzyme to the extent of 5 mcgm. of iron and 0.15 mcgm. of molybdenum per mgm. protein. Nitrate reductase activity in cell homogenates was decreased with increased oxygen tension but this effect was offset by pretreating the extract with carbon monoxide, presumably because the latter inhibited cytochrome oxidase. A possible sequence of electron transfer during nitrate reductase action in P. aeruginosa is:



A generalized scheme for nitrate reduction may therefore be outlined as follows:



Walker and Nicholas (1961a) have shown that Neurospora crassa wild type grown at low oxygen tension dissimilates nitrate and under these conditions nitrate reductase is dependent on iron as well as molybdenum for maximum activity.

At low oxygen tension the enzyme activity markedly increased, nitrite accumulated and cytochromes b and c increased in the felts. The results suggested that, when oxygen is limiting, nitrate is dissimilated by an active nitrate-reductase.

Pichinoty and D'Ornano (1961) found that during aeration of the Aerobacter aerogenes suspension, no trace of nitrite appears, but nitrite formation from nitrate, resumes its normal rate when aeration is stopped. This indicates that nitrate reductase activity is inhibited reversibly by oxygen, or in other words, aeration does not affect the stability of the enzyme. Extracts of cells induced under anaerobic conditions have significant activity. Non-induced cells and cells induced aerobically are completely inactive. On the other hand, cells which have been incubated aerobically synthesize as much enzyme as the anaerobic suspension when anaerobic conditions are commenced. This indicates that oxygen reversibly

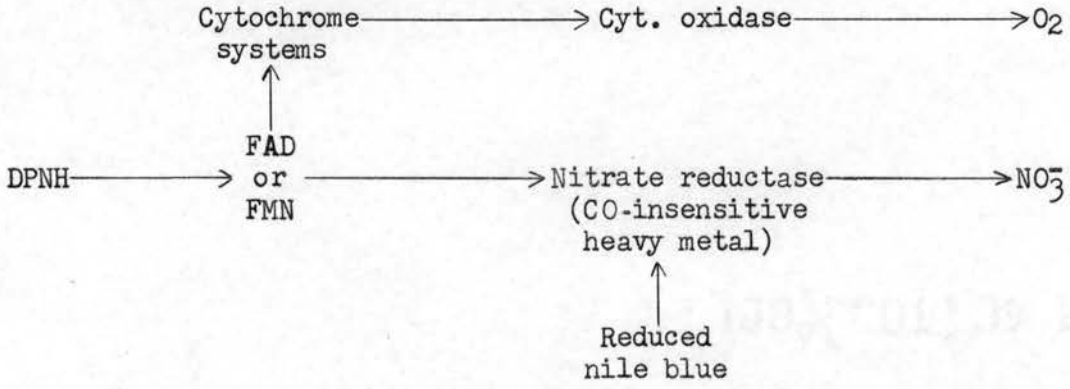
inhibits the biosynthesis of nitrate-reductase.

Taniguchi and Ohmachi (1960b) found inducible DPNH-nitrate reductase of sulfhydryl carbon monoxide insensitive metallo-enzyme in the large particles from nitrate-grown Azotobacter cells. The activity was stimulated 1.5- to 2-fold by externally added FAD or FMN.

Reduced nile blue was shown to be a most effective electron donor with 2- to 3-fold increase in the reaction rate from that of DPNH-nitrate reductase. The reduced nile blue-nitrate reductase activity was unaffected by FAD, FMN and p-chloromercuribenzoate.

In contrast to DPNH-oxidase in the large particles, DPNH-nitrate reductase has no participation of cytochrome components bound to the particle, though the latter system was inhibited by oxygen.

The scheme of electron-transferring sequence leading to nitrate apparently of nitrate assimilation type can be summarized as the following:



Woods (1938) found that washed suspensions of Clostridium welchii are able to catalyse the reduction of nitrate, nitrite, and hydroxylamine to ammonia by molecular H_2. During reduction of nitrate an appearance and final disappearance of nitrite can be demonstrated. This makes it clear that nitrite is an intermediate in the reduction of nitrate.

Nason and Evans (1953) reported that the purified nitrate reductase from Neurospora catalyzes the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) according to the equation:

$$NO_3^- + TPNH + H^+ \longrightarrow NO_2^- + TPN^+ + H_2$$

The maximal rate of activity achieved with TPNH was about 20-fold that obtained with DPNH. The equilibrium constant for the reaction is calculated to be 10^{27} and the reaction cannot be reversed. The enzyme which has been purified approximately 20-fold, has a pH optimum at 7.0 and is flavoprotein with FAD as prosthetic group as shown by reactivation studies. Inhibition by p-chloromercuribenzoate was reversed by cysteine or glutathione strongly suggested that sulfhydryl (-SH) groups are present on the enzyme. The enzyme is inhibited by cyanide, azide and thiourea, indicating a heavy metal constituent.

Nicholas et al. (1954a) found that cell-free extracts of molybdenum-deficient Neurospora crassa and Aspergillus niger showed a striking decrease in nitrate reductase, resulting in an enzyme level ranging from one-tenth to one-thirtieth of the controls. Molybdenum is required by the two fungi when nitrate is the source of nitrogen. Nicholas and Nason (1954b) identified molybdenum as the metal constituent of nitrate reductase. Their data show that the molybdenum level increased with the specific activity of the enzyme. Nicholas and Evans (1954c) from their studies of the mechanism of action of nitrate reductase from Neurospora showed that during the enzymatic transfer of electrons from TPNH to NO_3^- both FAD (or FMN) and molybdenum function as electron carriers. The reduction sequence mediated by tne enzyme is as follows:

$$TPNH + H + FAD \longrightarrow TPN^{+} + FADH_2$$

$$FADH_2 + Mo \longrightarrow FAD + "MoH_2"$$

$$MoH_2 + NO_3^{-} \longrightarrow Mo + NO_2^{-} + H_2O$$

Net reaction $$TPNH + H + NO_3^{-} \longrightarrow TPN^{+} + NO_2^{-} + H_2O$$

That flavin precedes molybdenum in this sequence was shown by the fact that cyanide-treated or molybdenum-free enzyme loses its ability to catalyze the reduction of NO_3^{-} to NO_2^{-} by TPNH or reduced flavin. Although it will catalyze the reduction of flavin with a concomitant oxidation of TPNH, the addition of Na_2MoO_4 specifically restores the ability of molybdenum-free enzyme to catalyze the formation of NO_2^{-} from NO_3^{-} by reduced flavin. The role of sulfhydryl (-SH) groups, which are necessary for the enzymatic transfer of electrons from TPNH to flavin is indicated by the marked inhibition by p-chloromercuribenzoate and its reversal by glutathione. Subsequent electron transport to nitrate is inhibited to a smaller extent by the mercury reagent.

Nicholas and Stevens (1955a) from their studies of valency changes of molybdenum during the enzymatic reduction of nitrate in Neurospora demonstrated that Mo^{5+} is as effective as reduced TPN as electron donor for the enzymatic reduction of NO_3^{-} to NO_2^{-}, however, Mo^{6+} is without effect. In the absence of nitrate reductase or in the presence of the boiled enzyme, Mo^{5+} does not reduce NO_3^{-}.

Nicholas and Nason (1955b) found that E. coli has a DPN-linked nitrate reductase with FAD and probably molybdenum as cofactor. It is likely that mechanism of electron transport is similar to that established for the enzyme in Neurospora, namely that the sequence of electron transport is as follows:

$$DPNH \longrightarrow FAD \text{ (or FMN)} \longrightarrow Mo \longrightarrow NO_3^{-}$$

The presence of -SH groups on the enzyme was indicated by the glutathione reversal of p-chloromercuribenzoate inhibition.

Nicholas and Scawin (1956) showed that the phosphate is required for nitrate reductase activity and can be replaced completely by selenate, tellurate or arsenate and to lesser extent by sulphate or silicate. It is likely therefore, that one of the roles of phosphate in nitrate reductase is to bind the molybdate to the enzyme.

Sadana and McElroy (1957) from their studies on highly purified nitrate reductase from Achromobacter fischeri, proposed that the pathway of electron transport for nitrate reduction in this microorganism is as follows:

$$\text{DPNH (or TPNH)} \longrightarrow \text{FMN (or FAD)} \longrightarrow Fe^{3+} \longrightarrow \underset{\text{cytochrome}}{\text{Bacterial}} \longrightarrow$$

$$\underset{\text{reductase}}{\text{Nitrate}} \longrightarrow NO_3^-$$

Silver and McElroy (1954) reported that enzyme studies on four strains of nitrate mutants of Neurospora have shown that in all cases the genetic block affected the apoenzyme of nitrate reductase, either directly or indirectly. In two strains no enzyme could be detected, another had detectable enzyme only when grown at pH 6.0 or greater, and the fourth strain, which has no nitrate reductase, produced a heat-labile inhibitor of wild type nitrate reductase. Enzyme and growth studies were made on five different types of nitrite mutants, comprising eight strains. Only one strain was found to have low nitrite reductase, whereas, three had low nitrate reductase. Two accumulated considerable hydroxylamine and nitrite in the culture medium. One strain was pH sensitive and required pyridoxine for growth. Under conditions of pyridoxine deficiency, NO_2^- accumulated and nitrite reductase activity was decreased.

Silver (1957) found that yeast nitrate reductase is a reduced pyridine nucleotide-linked metalloflavoprotein, with FAD and molybdenum as probably prosthetic groups. The enzyme is detected only in extracts of yeasts which utilize nitrate as a sole source of nitrogen.

Mendel and Visser (1959) found that the distribution of N^{15} followed the same pattern in all organs of intact plants, whether the nitrogen source was N^{15}-labelled ammonium or N^{15}-labelled nitrate. Plants which received ammonium contained the highest concentration of N^{15} in the roots, whereas those which received nitrate contained the highest concentration in the leaves.

Tomato leaf disks are able to assimilate nitrate in both the light and dark, but the rate of assimilation in the light is about 50 percent greater than in the dark. Carbohydrate depletion in leaf tissue results in a decreased nitrate reduction in the dark, whereas it is without effect in the light. Iodacetate markedly inhibits the reduction of nitrate in leaf disks incubated in the dark, but has no effect on the process in the light.

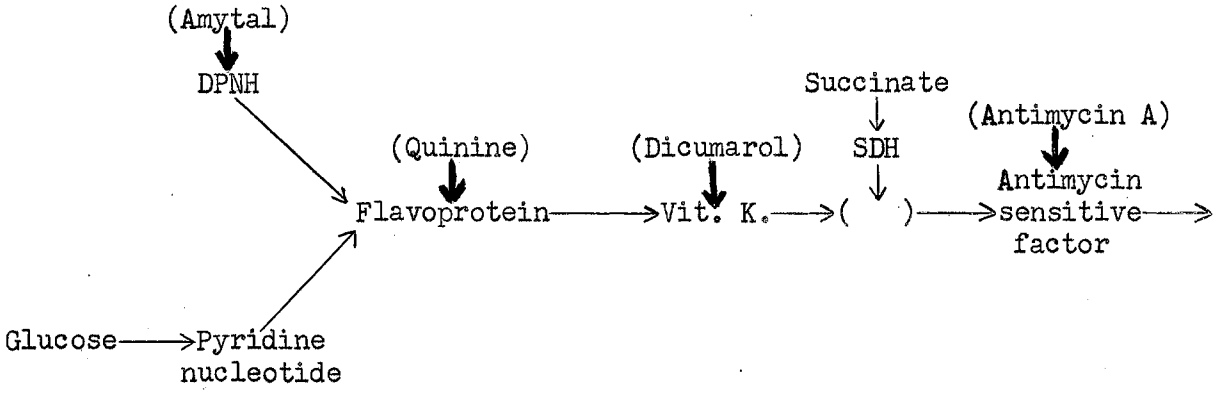
Nitrite. Asano (1959a) reported that electron transport system functioning in the reduction of nitrite and properties of nitrite reductase were studied using a halotolerant Micrococcus (strain 203) as the material.

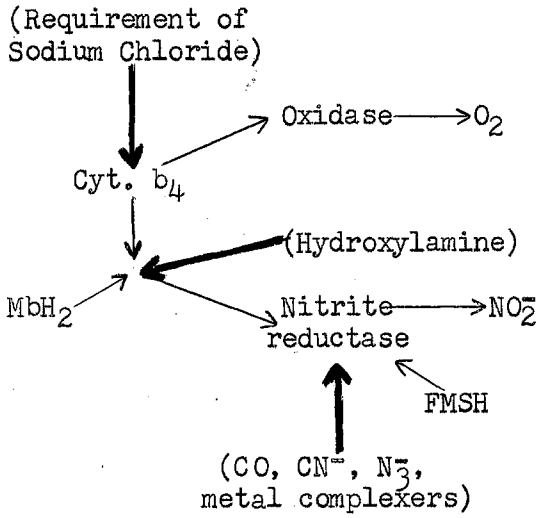
Ferrocytochrome reduced nitrite in the presence of nitrite reductase; the reaction required high salt concentrations. Electron transport from DPNH to nitrite, requiring high salt concentrations, was activated by FAD about 2-fold, and inhibited by antimycin A.

Glucose-nitrite reductase system was inhibited by amytal, quinine, dicumarol and antimycin A, and activated by FAD and menadione.

Succinate-nitrite reductase system was inhibited by antimycin A but not inhibited by amytal, quinine and dicumarol. Nitrite reductase was inhibited by cyanide, azide and metal chelators. The carbon monoxide inhibition in the dark was not reversed by light. Hydroxylamine inhibited the reduced methylene blue-nitrite reductase system but did not inhibit reduced phenazine methosulfase-nitrite reductase system.

The results described above may be summarized as follows:





Asano (1959b) stated that nitrite reductase of halotolerant Micrococcus (strain 203) was separated to soluble and particulate components by centrifugation. Each fraction did not show the nitrite reductase

activity by itself, but the activity appeared when both fractions were recombined. Different metals may be functioning in soluble and particulate fractions.

Asano (1960) reported that the particulate component of nitrite reductase of a halotolerant Micrococcus (strain 203) was solubilized by the treatment with snake venon and purified by ammonium sulfate fractionation. Using partially purified and thoroughly dialyzed preparations, it was found that the activity of soluble component is activated by Cu^{+} and Cu^{++} and that of particulate component is enhanced by Fe^{++} and Fe^{+++}.

Najjar and Allen (1954) reported that cell-free extracts which reduce nitrite to a mixture of gaseous products have been obtained from Pseudomonas stutzeri and a denitrifying Bacillus subtilis strain. The gas formed by P. stutzeri extracts is principally nitrogen while B. subtilis preparations produce an appreciable quantity of nitrous oxide. Both form nitric oxide from nitrite. Nitric oxide can be reduced to nitrogen by extracts and by intact cells. Iwasaki et al. (1956) found that nitric oxide is produced from nitrite and is utilized in place of the latter and it is presumably assumed that nitric oxide is an intermediate in denitrification.

Chung and Najjar (1956a) found that cell-free extracts of Pseudomonas stutzeri were capable of producing nitric oxide and nitrogen from nitrite. Ammonium sulfate fractions showed marked stimulation of activity when DPN and TPN was added in catalytic amounts with the necessary hydrogen donor substrates. FAD and FMN also stimulated definite nitrite reductase. Of the metal ion tested only Cu^{++}, Fe^{++}, and Fe^{+++} produced definite stimulation.

Spencer et al. (1957) showed that nitrite and hydroxylamine

reductases utilize reduced pyridine nucleotide as electron donor and require added flavin for maximal activity, FAD specifically in the case of nitrite reductase and FAD or the FMN with hydroxylamine enzyme. Inhibitor studies indicate that the systems have an essential metal component. Mn^{2+} was demonstrated to be specific activator of hydroxylamine reductase. The product of the reduction of NO_2^- by these extracts was identified as ammonia. Approximately one molecule of ammonia is formed for each mole of NO_2^- utilized. The enzyme has a pH optimum at 7.1.

Medina and Nicholas (1957) showed that nitrite, hyponitrite and hydroxylamine reductases in Neurospora to be metalloflavoprotein enzymes, the first two require iron and copper and the last one is manganese dependent, based on metal deficiency and inhibitor studies. Uncoupling reagent depressed nitrite and hyponitrite reductases but were without effect on hydroxylamine reductase.

Nicholas et al. (1960) reported that nitrite reductase from wild type N. crassa has been purified over 50-fold. The enzyme is a DPNH-dependent, flavoprotein, containing FAD, iron and copper and -SH groups. Copper may act by coupling the flavin component of the enzyme to NO_2^-, since an external supply of Cu^+ reduced NO_2^- non-enzymatically, the reduction is increased in the presence of the enzyme. The role of iron in the enzyme is not known since neither Fe^{++} nor reduced cytochrome c will reduce NO_2^-. The enzyme is also dependent on magnesium and pyridoxine for maximal activity. The reduction product inhibited the enzyme and it is shown that the addition of hyponitrite but not of NH_2OH depressed nitrite reductase activity. The enzyme maximal activity was reached at about pH 7.6 in phosphate buffer.

Walker and Nicholas (1961b) reported that a nitrate reductase was

purified more than 600-fold from extracts prepared from actively denitrifying Pseudomonas aeruginosa. The enzyme reduced nitrite to nitric oxide when either flavins (Riboflavin H_2, $FMNH_2$, $FADH_2$) reduced pyocyanine, reduced methylene blue or reduced 1, 4-naphtho-quinone was the electron donor. DPNH, TPNH and reduced cytochrome c were ineffective as donors. The stoichiometry of this reaction was determined as follows:

$$PyH_2 + 2NaNO_2 \longrightarrow 2NO + Py + 2NaOH$$

The enzyme contained 1.5 millimicromoles FAD per mgm. protein and FAD was required for maximum enzyme activity when reduced pyocyanine was the donor but not when reduced methylene blue was used. The enzyme has a cytochrome c type spectrum and an additional maximum between 630 and 635 mμ which is probably associated with copper. The latter was reduced by electron donors that were effective for the purified enzyme and reoxidized specifically by nitrite. Metal deficiency studies and the accumulation of the metal in the enzyme indicate that iron and copper are required for nitrite reductions. The enzyme required -SH groups and either phosphate or sulphate for maximum activity. The sequence of electron transfer in the enzyme is probably as follows:

$$PyH_2 \longrightarrow FAD \longrightarrow \underset{\uparrow \; MbH_2}{\overset{(1^+ \rightleftharpoons 2^+)}{\text{Cu-protein}}} \longrightarrow NO_2^-$$

Nitric Oxide. Chung and Najjar (1956) reported that nitric oxide reductase was obtained from extracts of Pseudomonas stutzeri which were fractionated with ammonium sulfate. The enzymatic reduction of nitric oxide to nitrogen required the presence of a pyridine and a flavin nucleotide as well as copper for maximal activity. The enzyme contains substantial amounts of cytochrome c. That this compound plays a part in the

electron transfer during denitrification is strongly suggested by its accumulation in large quantities during anaerobic growth of the organism in the nitrate or nitrite medium. Furthermore, it was found that intact cells show an absorption band at 556 mμ which disappears when the cells are exposed to nitrate or nitrite.

The electron transfer mechanism may be visualized as follows:

$$H^{+} + \frac{TPNH}{DPNH} \longrightarrow FAD \longrightarrow Cu^{++} \text{ and/or } Fe^{+++} \text{ (Cyt. c.)} \longrightarrow No$$

Fewson and Nicholas (1960) reported that nitric oxide reductase purified 25-fold from P. aeruginosa. The activity of the purified enzyme, which utilized reduced pyocyanine but not reduced pyridine nucleotides as the hydrogen donor, was maximal at pH 8.0; sulfhydryl groups appear to be required for the enzyme activity since glutathione partially affects the inhibition by p-chloromercuribenzoate. A flavin requirement for the enzyme was indicated by the reversal of mepacrine inhibition with FMN or FAD.

They also concluded from the results presented in their paper that nitric oxide, or a compound with which it equilibrates, is an intermediate in both assimilatory and dissimilatory nitrate reduction. Based this on two main lines of evidence: (1) Nitric oxide is the immediate product of nitrite reduction in Neurospora and in several denitrifying bacteria, and (2) nitric oxide reductase activity is found in microorganisms and higher plants which are actively reducing nitrate, but the enzymes are absent or much reduced in organisms grown on ammonium-nitrogen only.

Inhibition of Hydrogenase Activity by Nitric Oxide in Vitro. Krasana and Rittenberg (1954) found that nitric oxide at low concentrations inhibits hydrogenase activity of Proteus vulgaris.

Atkinson (1955) has shown that nitrate in the growth medium at 0.03

to 0.05 M strongly decreases the hydrogenase activity of heterotrophically or autotrophically grown Hydrogenomonas facilis. The simple inorganic nitrogen compounds intermediate in oxidation between nitrate and ammonia were tested for inhibition of the hydrogenase (methylene blue assay) and of the hydrogen-oxygen reaction. Nitrate, nitrite, nitrous oxide, and hydroxylamine are without effect on methylene blue reduction even at high levels; of these, only hydroxylamine inhibits oxygen reduction significantly. The strongest effect on methylene blue reduction is exerted by nitric oxide, which causes 50 percent inhibition at about 3×10^{-5} M.

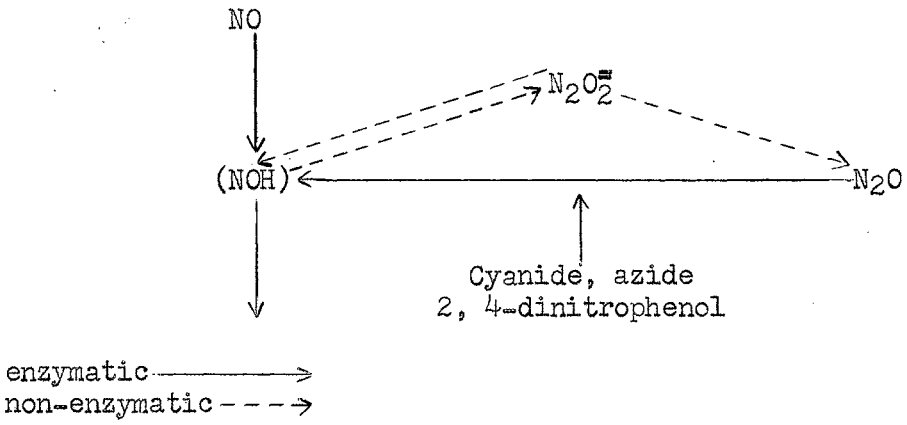
Hyponitrite, Nitroxyl and Nitrous Oxide. Sacks and Barker (1952) found that denitrifying resting cells of Pseudomonas denitrificans can reduce nitrous oxide to nitrogen, but a lag period is often observed before the conversion commences. Suitable concentrations of azide and dinitrophenol inhibit the utilization of nitrous oxide without affecting the reduction of nitrite to nitrogen. This is considered evidence that nitrous oxide cannot be the normal precursor of nitrogen in the reduction of nitrite to nitrogen.

Allen and Van Neil (1952) reported that application of the technique of simultaneous enzymatic adaptation with P. stutzeri suggested that nitrous oxide either is an intermediate product in denitrification or is reversible derived from an intermediate product. Studies on the effect of cyanide on the reduction of nitrate, nitrite, and nitrous oxide revealed that nitrite can still be converted into gaseous products under conditions where reduction of nitrate and nitrous oxide is completely prevented by cyanide. The gas which is produced from nitrite under these conditions is nitrogen, not nitrous oxide, hence nitrous oxide cannot be

an intermediate product in denitrification.

Delwiche (1959) reported that a lag was observed in the utilization of nitrous oxide by cells of Pseudomonas denitrificans grown in the presence of oxygen or of nitrate ion. Cells grown on oxygen as a hydrogen acceptor exhibited a longer lag period than that for nitrate.

Fewson and Nicholas (1961c) integrated the results of the above workers by assuming that a compound like nitroxyl (NOH), containing one nitrogen atom, is the intermediate in nitrate reduction, and that this is in equilibrium with hyponitrite which can decompose to give nitrous oxide. A further adaptive enzyme is necessary to utilize nitrous oxide. This is illustrated in the following scheme:



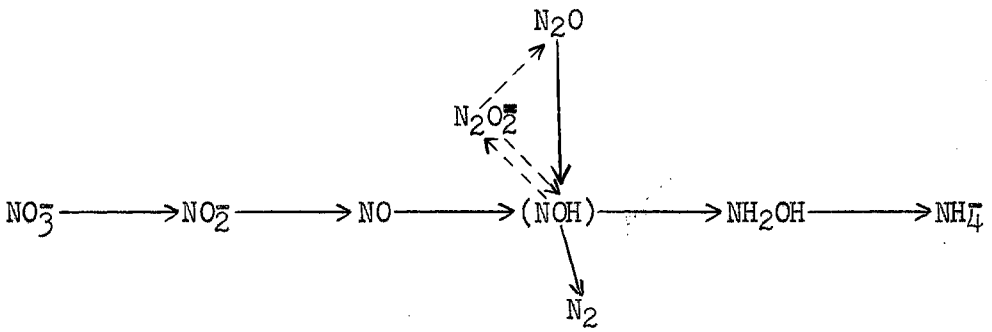
The scheme is supported by the following lines of evidence:

1. Nitrous oxide has frequently been reported as a product of denitrification.

2. This scheme explains the utilization of hyponitrite, since it would exist in equilibrium with nitroxyl and would also produce nitrous oxide which could be utilized.

3. The true intermediate is likely to contain only one nitrogen atom, as do other intermediates.

They also suggested the following scheme for both assimilatory and dissimilatory reduction in microorganisms:



enzymatic ——→
non-enzymatic - - →

Hydroxylamine. Zucker and Nason (1955) reported that a soluble enzyme system which reduced hydroxylamine to ammonia has been purified 5- to 10-fold from cell-free extracts of Neurospora crassa. Both DPNH and TPNH serve as electron donors in the reaction. FAD but not FMN stimulates the activity of the enzyme 2- to 3-fold and metal chelating agents inhibit the activity of the enzyme, KCN being the most effective, suggesting a metal constituent. Stoichiometry among hydroxylamine disappearance, DPNH as well as NH_3 production, and DPN^+ formation support the following equation for the reaction:

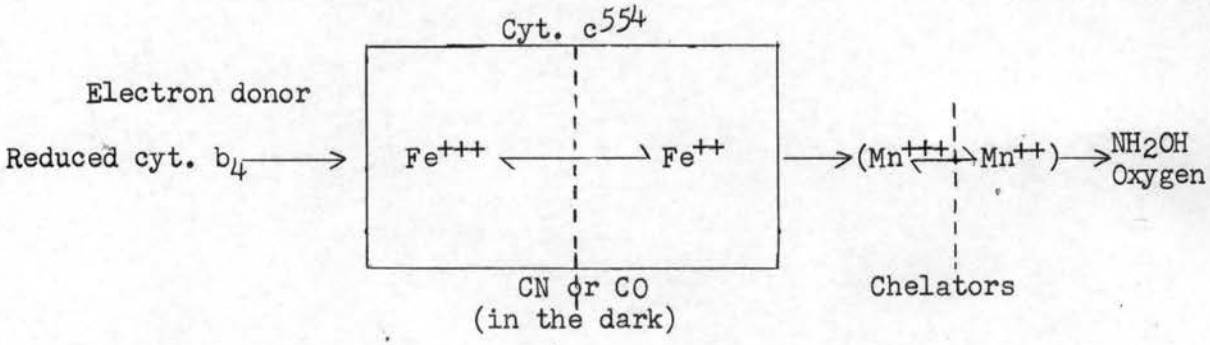
$$NH_2OH + DPNH + H^+ \longrightarrow NH_3 + DPN^+ + H_2O$$

The reaction has not been reversed. The enzyme is adaptive, forming only in the presence of nitrate, or nitrite but not ammonia. These data suggest that NO_3^- can give rise to hydroxylamine and subsequently to ammonia in Neurospora.

Kono and Taniguchi (1960) reported that a hydroxylamine reductase of hemoprotein nature was prepared from a strain of halotolerant Micrococcus. The hydroxylamine reductase was shown to contain iron as its active center. Manganese ions at 0.5 mM concentration activate the reaction about three to four times when the activity is measured by the

reduced methylene blue method; these ions possibly participate in the formation of a hydroxylamine reductase-hydroxylamine complex probably with a simultaneous valency change. The reduced form of this hemoprotein is oxidized by both oxygen and hydroxylamine, the reactions being similarly activated by manganese ions. Carbon monoxide combines with the reduced hemoprotein photoreversibly. Pyridine hemochromogen from this enzyme has the same absorption maxima as that from mammalian cytochrome c. This autoxidizable and carbon monoxide-binding cytochrome c (its reduced alpha-peak is at 554 mμ) which can be obtained from the halotolerant Micrococcus is shown to be hydroxylamine reductase. The above findings are summarized schematically in the following scheme:

Micrococcus hydroxylamine reductase



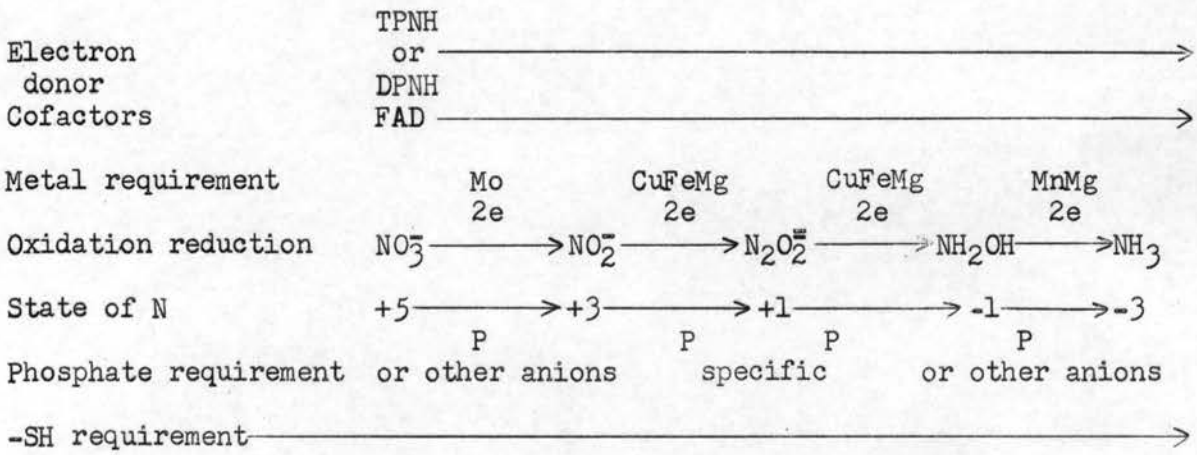
Walker and Nicholas (1961c) reported that hydroxylamine reductase was purified 47-fold from extracts of actively denitrifying P. aeruginosa. The enzyme reduced hydroxylamine to ammonia when either reduced pyocyanine or reduced methylene blue was the donor. The enzyme contained FAD which could be partly removed by ammonium sulphate precipitation at pH 6.0. The apoenzyme was reactivated by FAD but FMN was without effect. The felts deficient in the metal had reduced activity. The purified enzyme when first dialyzed against 10^{-3} M sodium diethyldithiocarbamate and then

phosphate buffer, to remove the chelating agent, was activated by manganese. Although cobalt partly substituted for manganese it was only 10 percent as effective.

When purified hydroxylamine reductase was used and the stoichiometry of either MBH_2 or PYH_2 oxidation and hydroxylamine reduction and ammonia production followed, it was found that for every mole of electron donor oxidized a mole of hydroxylamine was reduced. The stoichiometry of the reaction is therefore:

$$NH_2OH + PyH_2 \text{ or } MbH_2 \longrightarrow Py \text{ or } Mb + NH_4OH$$

Nicholas (1959) reported that a series of metallo-flavoprotein enzymes has been characterized in plants which reduced nitrate to ammonia. The main feature of inorganic route may be briefly summarized as follows:



Metabolism of Nitrate by Animals

Lewis (1951a) reported that nitrate introduced into the rumen of the sheep is reduced to ammonia. Nitrite is an intermediate in this reaction and may accumulate under certain conditions and lead to a partial conversion of blood hemoglobin to methemoglobin. Sodium nitrate (25 gm.) or sodium nitrite (10 gm.) placed in the rumen, or 2 gm. sodium nitrite

injected intravenously, results in a methemoglobinemia corresponding to 60 percent conversion of the total hemoglobin.

Lewis (1951b) reported that hydrogen is a very active donator for the reduction of nitrate, nitrite and hydroxylamine to ammonia, in the presence of washed microorganisms from sheep rumen.

Nitrite is an intermediary in the reduction of nitrate, and appears transitorily in the early stages of reduction by hydrogen.

The pH optima of nitrate and nitrite reduction in an atmosphere of hydrogen are 6.5 and 5.6, respectively, whereas the optimum for hydroxylamine reduction shows a broad plateau over the range of pH six to seven. Formate, succinate, lactate, citrate, glucose, malate and mannitol are hydrogen donators for nitrate reduction, but at lower rates than hydrogen. Glycerol, xylose and ethanol have little activity and acetate, propionate and n-butyrate are inactive.

O'Dell et al. (1960) studied the effect of nitrite addition upon vitamin A status of rats. Group I received vitamin A deficient basal diet and Group II received the basal diet plus 0.3 percent potassium nitrite. A period of six to eight weeks was required for depletion. At this time xerophthalmia was evident in Group II and growth in both groups had stopped. Rats in Group I grew more rapidly than those of Group II. Liver vitamin A content was correlated with the growth data.

Wyngaarden (1952) found that perchlorate, periodate and nitrate shared with thiocyanate the properties of inhibiting collection and of interfering with retention of iodine ion within the thyroid glands of rats chronically treated with prophylthiouracil; concentrations of 10^{-4}M of thiocyanate, perchlorate, and nitrate resulted in discharging 100, 100 and 60 percent, respectively, of iodine previously collected in the

thyroid glands.

Johnson and Raumann (1947) found that rats receiving thiourea or thiouracil stored very little vitamin A. The administration of thyroxine neutralized the effects of both thiourea and thiouracil and restored the ability of the animals to convert carotene to vitamin A.

Drill and Truant (1947) found that supplements of vitamin A administered to rats receiving a diet devoid of vitamin A prevented the appearance of xerophthalmia in both controls and thyoidectomized animals; however, supplementation of carotene prevented the ocular changes only in the control rats and not in thyroidectomized rats. The mortality and changes in weight of the thyroidectomized animals receiving carotene also indicated a decreased utilization of carotene. It is apparent that the thyroid gland plays a major role in the conversion of carotene to vitamin A.

Wang et al. (1961) found that a cow receiving a direct addition of 70 to 100 gm. of potassium nitrate a day into the rumen through a fistula, showed no methemoglobin in the blood for several days. This delay in methemoglobin formation could not be attributed to failure of the microorganisms of the rumen to form nitrite, for they immediately showed vigorous nitrate reduction. Potassium nitrate containing N^{15} was added to the rumen for tracing the formation, absorption and elimination of nitrate and the compounds formed from it. Nitrate, nitrite and ammonium ions were absorbed in considerable quantities directly from the rumen into the blood. The highest concentrations of N^{15} of the ammonium ion in the blood appeared three to four hours after potassium (N^{15}) nitrate was added to the rumen, whereas the highest N^{15} concentration of nitrate N^{15} plus the nitrite fraction appeared in the blood after five to six hours,

and the high concentration persisted.

The curves for excretion of the N^{15}-labelled total nitrogen, nitrate, urea and ammonium nitrogen fractions in the urine lagged about an hour behind their corresponding curves for appearance and disappearance of these components in the blood. The formation of methemoglobin in the blood followed the time course of nitrite formation in the rumen rather closely, indicating that nitrite was passed rapidly and directly from the rumen to the blood. There was no direct evidence for nitrite formation from nitrate in the blood. Nitrite was bound tightly to the heme of methemoglobin, but could be recovered from heme that had been subjected to treatment with acetone containing 1.2 ml. of 12-N hydrochloric acid per 100 ml.

Nitrate toxicity has been observed frequently in ruminants fed on a variety of plant material high in nitrate such as oat hay or straw, and referred to as "oat poisoning", was reported by (Bradley et al., 1939; Newson et al., 1937; Savage, 1949; Whitehead et al., 1952). Sund et al. (1957) reported that a non-contagious abortion observed among cattle pastured on March land in Central Wisconsin apparently was a manifestation of nitrate toxicity. Lewis (1951) demonstrated that microorganisms in the rumen of sheep reduce nitrate to ammonium ions with nitrite as an intermediate and that enough nitrite was transferred to the blood to convert a considerable fraction of the hemoglobin into methemoglobin. Wang et al. (1961) showed that microorganisms in the rumen of cattle reduce N^{15}-labelled nitrate to ammonium ions with nitrite as an intermediate. Nitrate, nitrite, and ammonium ions were absorbed in considerable quantities directly from the rumen into the blood. The present investigations were designed to provide information concerning (a) the role

of molybdenum and iron in the reduction of nitrate to nitrite *in vivo* in the rumen of the sheep, and that of iron and copper in the further reduction of nitrite to ammonia, and (b) to study the time-course of nitrate reduction in the rumen, and the appearance of nitrate and nitrite in the blood.

EXPERIMENTAL PROCEDURE

Trial I. The composition of the purified diet, and the mineral mixtures used in this first trial are shown in Tables I and II. Nine native western-type lambs, averaging 53 pounds, were alloted equally and randomly to three treatments, and fed the basal ration (ration 1), the basal plus molybdenum (ration 2), and the molybdenum-supplemented ration minus both iron and copper (ration 3). All sheep were individually-housed in wooden stalls, which were designed to eliminate possible trace mineral contamination. The stalls, which were raised 30 inches from the ground, had wooden floors through which were bored many holes three-fourths of an inch in diameter, allowing for disposal of feces and urine. Distilled water, after having passed through a Barnstead Demineralizer containing cation and anion beds, was supplied to the animals in plastic containers. The feed was dispensed once daily in feeders made of wood. Blood samples were taken from the jugular vein at the intervals shown in Figures 1 and 2 during the experiment, and clotting was prevented by sodium oxalate. Oxalated blood was centrifuged within an hour to recover plasma and the protein-free blood filtrate was prepared from the plasma by the method of Folin and Wu (1919). The analyses for nitrate and nitrite in the protein-free blood filtrate were made by the methods described by Nicholas and Nason (1957).

TABLE I

PERCENTAGE COMPOSITION OF THE PURIFIED RATION (TRIAL I)

Ingredient	Percentage
Solka Floc	31.8
Starch	24.0
Cerelose	24.1
Mineral Mixtures	6.5
Potassium Nitrate	6.25
Sodium Nitrate	6.25
Corn Oil	1.0
Choline Chloride	0.1
Vitamin Supplement	
Vitamin A Acetate*	0.40 gm.
Calciferal (granular)**	0.05 gm.

*Vitamin A Acetate containing 1 million units per gram.

**Calciferol (granular) containing 850,000 units per gram.

TABLE II

PERCENTAGE COMPOSITION OF THE MINERAL MIXTURES (TRIAL I)

Ingredient	Ration 1	Ration 2	Ration 3
K_2CO_3	31.32	31.30	32.25
$CaHPO_4$	48.94	48.94	48.94
$MgSO_4$	10.75	10.75	10.75
NaCl	7.43	7.43	7.43
$FeSO_4$	0.91	0.91	-----
$MnSO_4$	0.11	0.11	0.11
$Na_2B_4O_7$	0.35	0.35	0.35
$CuCO_3$	0.04	0.04	-----
$ZnSO_4$	0.124	0.124	0.124
$CoCl_2$	0.0006	0.0006	0.0006
$Na_2MoO_4 \cdot 2H_2O$	------	0.0154	0.0154
KI	0.03	0.03	0.03

During the last 11 days of experiment, the animals of the three treatments were fed the molybdenum supplemented basal ration (ration 2).

Trial II. The rations fed during the second trial are shown in Tables III and IV. The animals which were used in this phase were two Hampshire-Western Cross, two-year-old wethers equipped with permanent rumen fistula. They were kept in metabolism cages and provided with feed and distilled water. The feed was dispensed in the feeders made of wood once daily for about 10 days prior to the start of the experiment; this is referred to as the conditioning period. One sheep was fed the basal ration (ration 1) and the other sheep fed the basal ration plus 10 ppm molybdenum (ration 2).

After the conditioning period, 500 gm. of each diet which contained 5 percent KNO_3 and 5 percent $NaNO_3$ was added through the rumen fistula of each sheep after being suspended in two liters of distilled-demineralized water. Samples of rumen fluid were withdrawn each hour (Figures 3-10) through the fistula and strained through six layers of cheese cloth. It then was clarified by the method of Lewis (1951), and the clear supernatant solution was used for analysis. Blood samples were removed from the jugular vein and the protein-free blood filtrate was prepared for analysis by the method of Folin and Wu (1919). Analyses for nitrate and nitrite in the rumen fluid and blood were made by the methods described by Nicholas and Nason (1957). Ammonia in the rumen fluid was determined by the method described by Conway (1950). The pH of rumen fluid samples was determined using the combination electrode of a Beckman Model N pH Meter.

TABLE III

PERCENTAGE COMPOSITION OF THE PURIFIED RATION (TRIAL II)

Ingredient	Percentage
Solka Floc	30.0
Starch	26.0
Cerelose	26.9
Mineral Mixtures	5.0
Potassium Nitrate	5.0
Sodium Nitrate	5.0
Polyethylene Resin	1.0
Corn Oil	1.0
Choline Chloride	0.1
Vitamin Supplement	
Vitamin A Acetate*	0.40 gm.
Calciferol (granular)**	0.05 gm.

*Vitamin A Acetate containing 1 million units per gram.

**Calciferol (granular) containing 850,000 units per gram.

TABLE IV

PERCENTAGE COMPOSITION OF THE MINERAL MIXTURES (TRIAL II)

Ingredient	Ration 1	Ration 3
K_2CO_3	44.38	44.33
$CaHPO_4$	20.00	20.00
Na_2SO_4	14.00	14.00
$MgSO_4$	10.00	10.00
NaCl	10.00	10.00
$FeSO_4$	0.85	0.85
$Na_2B_4O_7$	0.25	0.25
$MnSO_4 \cdot H_2O$	0.228	0.228
$ZnSO_4 \cdot 7H_2O$	0.2673	0.2673
$CuCO_3$	0.02	0.02
$Na_2MoO_4 \cdot 2H_2O$	-----	0.05
CaF_2	0.004	0.004
$CoCl_2 \cdot 6H_2O$	0.0009	0.0009
Na_2SeO_4	0.0005	0.0005
KI	0.0003	0.0003

RESULTS

Trial I. This trial was concerned with the metabolism of nitrates in the rumen and the appearance of nitrate and nitrite ions in blood. Figures 1 and 2 show, respectively, nitrate and nitrite ion concentration in blood plasma of sheep during 13 sampling intervals of the 52-day experiment.

During the first 10 intervals, the nitrate ion concentration was higher in blood plasma of sheep receiving the basal ration (ration 1) than those fed the basal ration plus molybdenum (ration 2) or the basal ration plus molybdenum minus both iron and copper (ration 3). When the values of the first 10 intervals were averaged, it was found that the nitrate ion concentration was 3.6- and 4.8-fold higher in blood plasma of sheep receiving ration 1 than those fed rations 2 and 3, respectively. In contrast, the nitrite ion concentration was the lowest in blood plasma of sheep on ration 1 than those on rations 2 and 3, respectively. The average of nitrite ion concentrations in blood plasma of sheep on ration 1 was 8.0- and 5.0-fold lower than of those on treatments 2 and 3, respectively.

The nitrate and nitrite ion concentrations during the first 10 intervals were slightly higher in blood plasma of sheep fed ration 2 than those receiving ration 3. These differences might be accounted for by differences in daily feed consumption; the averages of daily feed consumption were 0.73, 0.66 and 0.33 pound per sheep of the treatments 1, 2 and 3, respectively.

During the last 11 days of experiment, all sheep received the basal ration plus molybdenum (ration 2). Figures 1 and 2, respectively, show

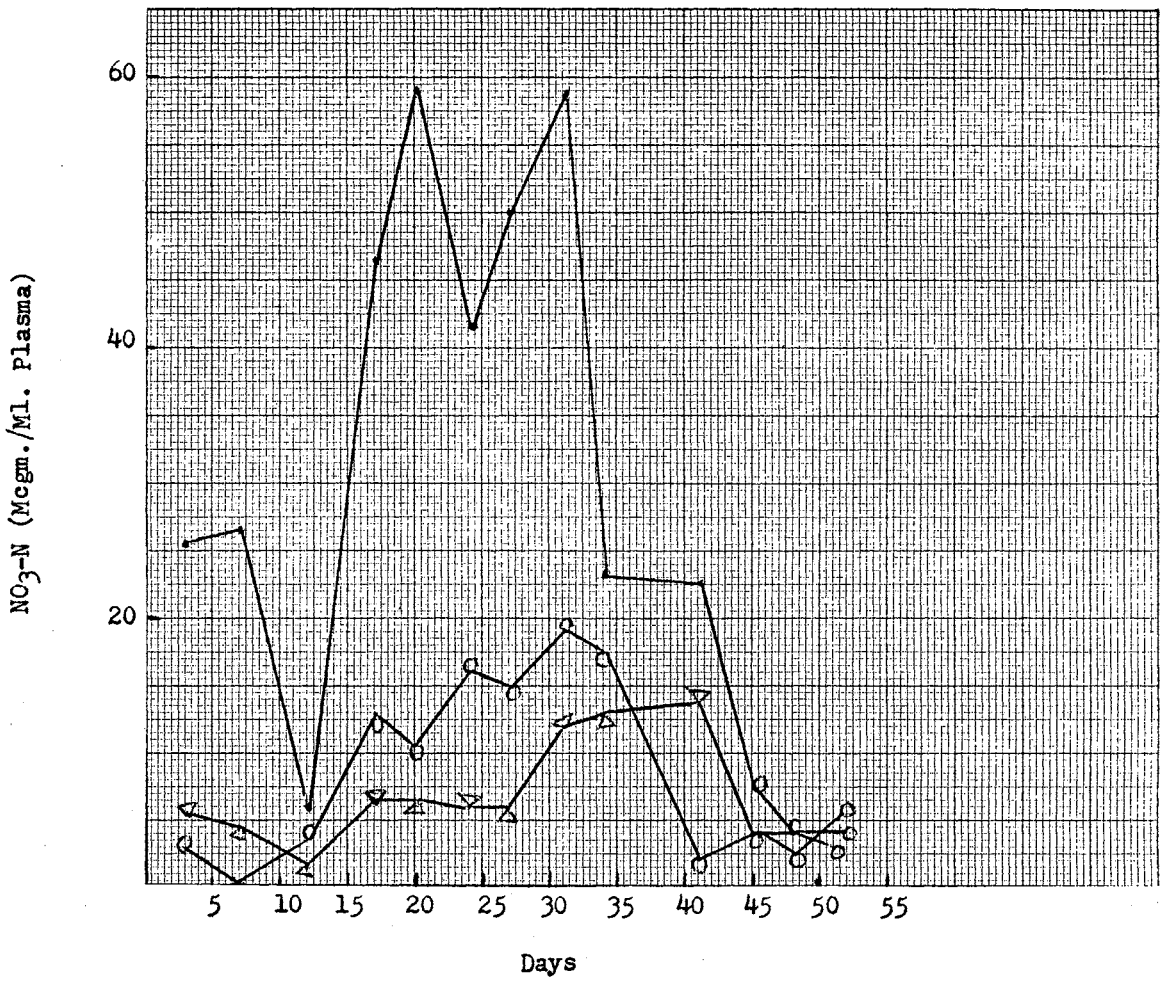


Figure 1. Nitrate-Nitrogen in Blood Plasma of Sheep Fed Purified Rations Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

Δ Basal Plus Molybdenum, Minus Both Copper and Iron

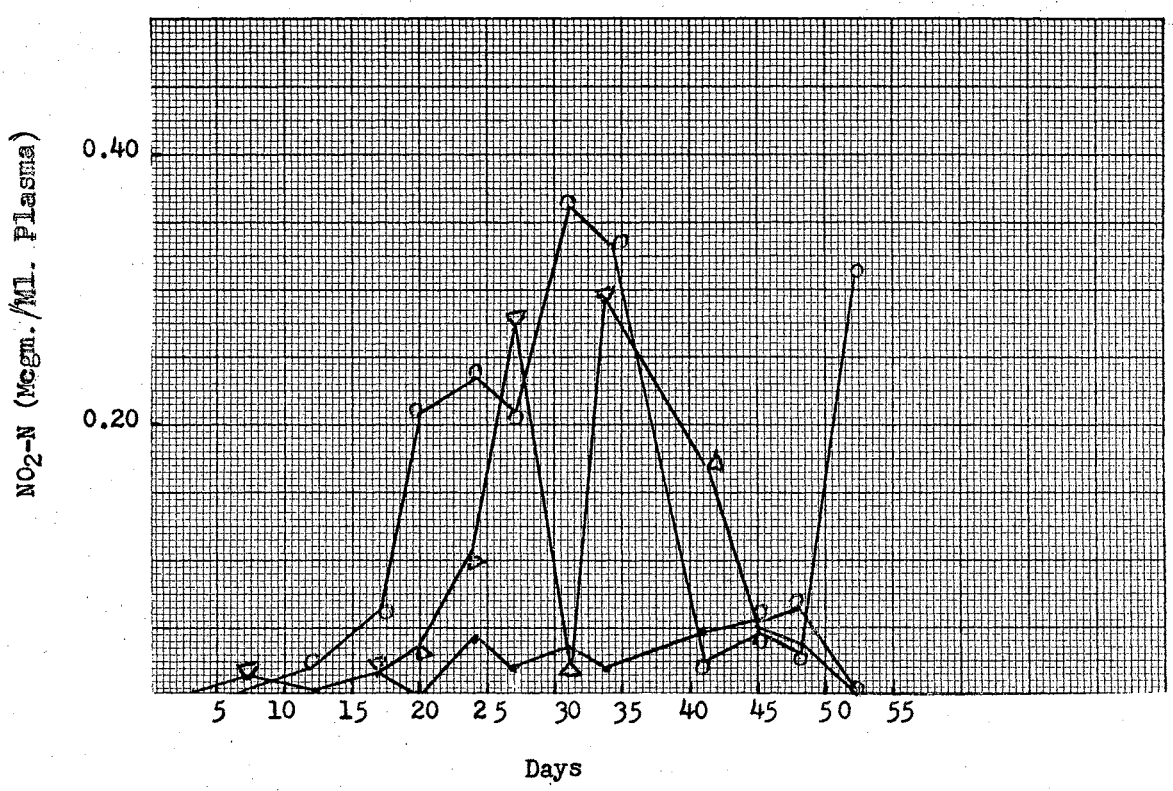


Figure 2. Nitrite-Nitrogen in Blood Plasma of Sheep Fed Purified Rations Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

Δ Basal Plus Molybdenum, Minus Both Copper and Iron

no differences in nitrate and nitrite ion concentration between the blood plasma of all the sheep during these last three intervals, with the exception of the thirteenth interval, during which time the sheep which had, during the previous 10 intervals received the same diet (ration 2), showed the higher plasma nitrite ion concentrations than those which had been on rations 1 and 3 during the first 10 intervals. The average daily feed consumption during the last 11 days was 0.46, 1.0 and 0.59 pounds per sheep for the sheep which had previously received rations 1, 2 and 3, respectively. In other words, sheep which had previously received ration 2 ate about twice as much feed as those that had previously been on rations 1 and 3.

Trial II. The concentrations of nitrate, nitrite, ammonium, and hydrogen ions in rumen liquid, as well as the concentrations of the first two in blood, were followed periodically in two sheep after 500 gm. of basal ration or basal ration plus molybdenum, suspended in two liters of distilled and demineralized water, was administered through the fistula. During the first four hours the rumen pH fell gradually from 7.4 and 7.2 to 5.6 (Figure 3) in the rumen of sheep on rations 1 and 2, respectively. After that time, it remained constant in the control sheep with slight decrease in the last hour of sampling, while in other sheep it started rising steadily until it reached 6.5 during the succeeding three hours, then fell to 6.4 and 6.2 during the eighth and ninth hour of sampling, respectively.

The effects of treatments upon rumen nitrate concentration are shown in Figure 4 and it may be seen that nitrate disappeared from the rumen of the sheep fed the molybdenum-supplemented diet at 1.3- to 2.1-fold faster rate than that of sheep fed the basal diet containing no

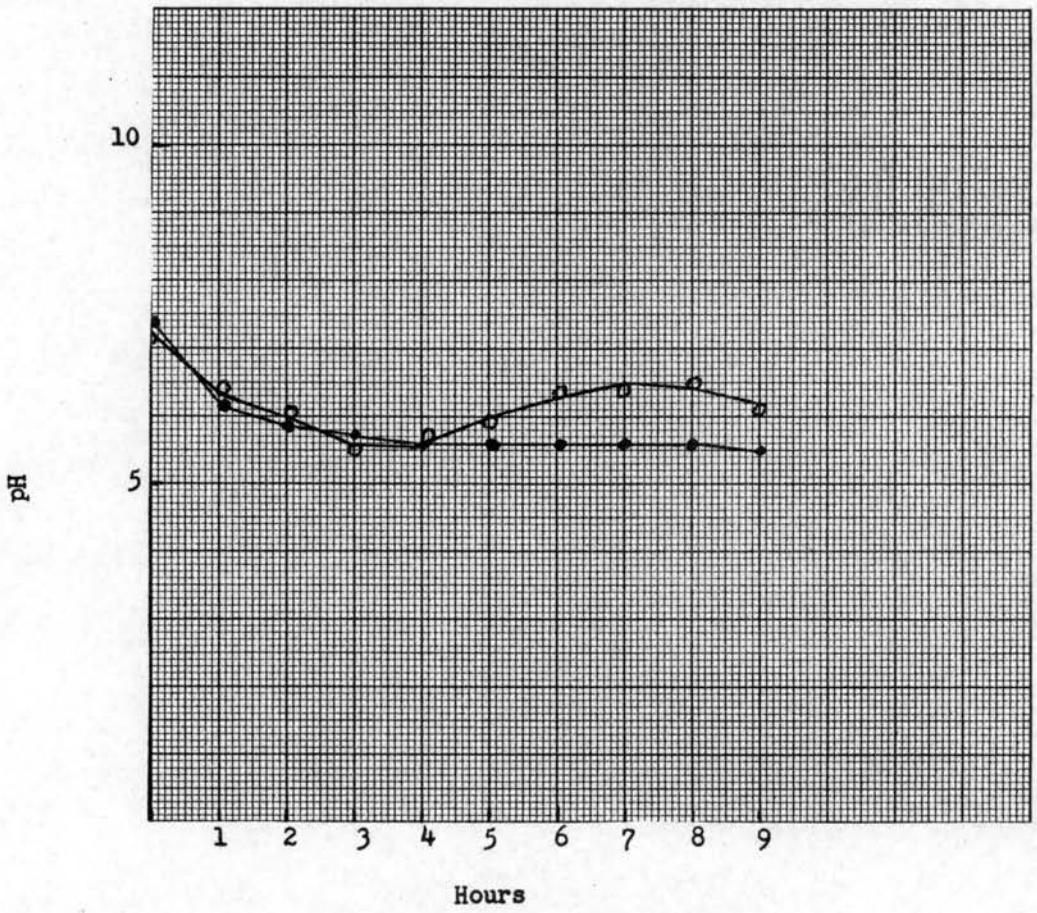


Figure 3. pH Readings of Rumen Fluid of Sheep After Administration of Diets Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

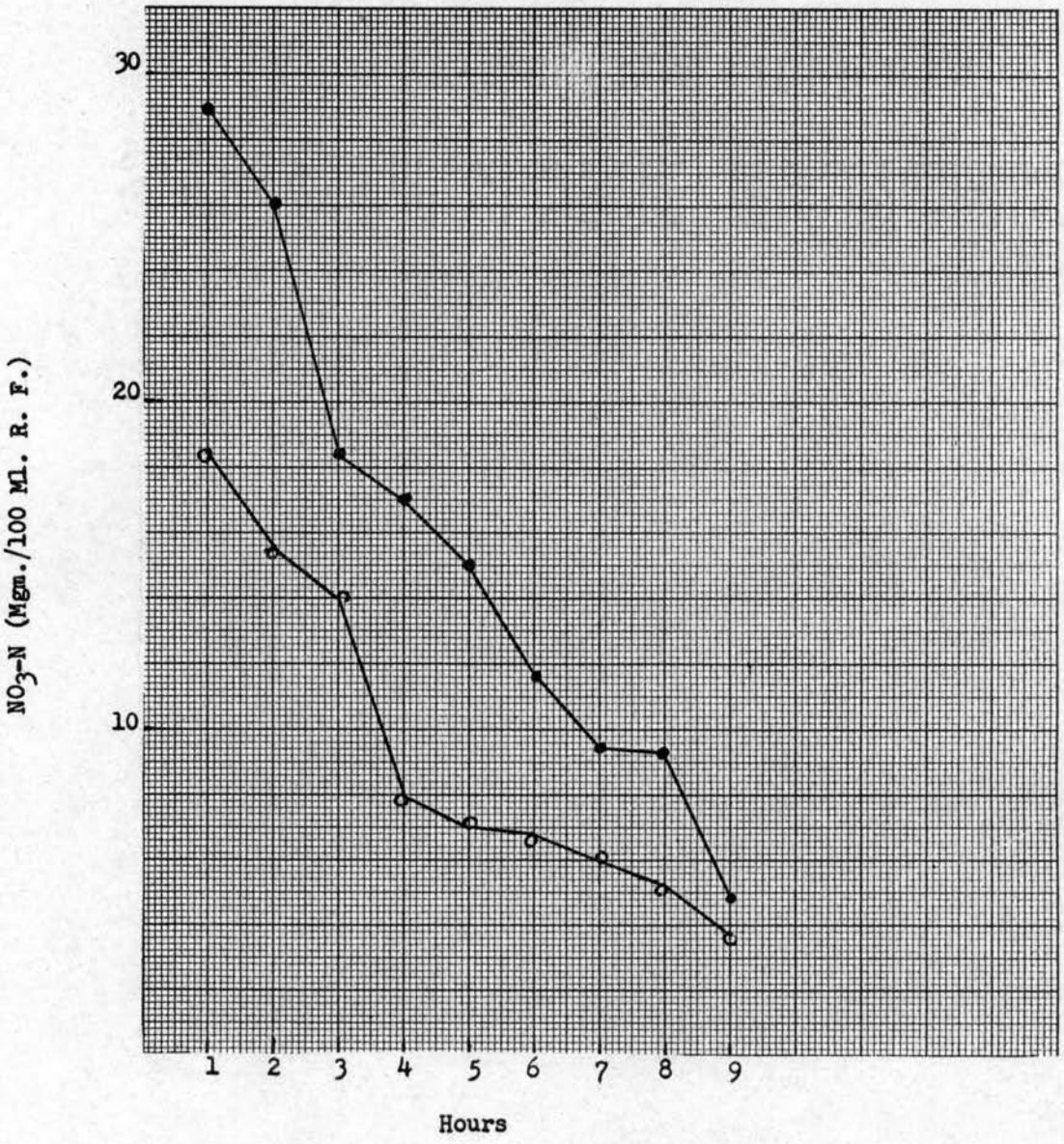


Figure 4. Nitrate-Nitrogen in Rumen of Sheep After Administration of Diets Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

molybdenum.

Nitrite production (Figure 5) coincident with nitrate disappearance rose sharply in the treated sheep and steadily in control sheep until it reached maximum concentration at the third and fourth hours, respectively, after administration of the nitrate-containing diet, and then fell at decreasing rate in the former and uniformly in the latter during the next six and five hours, respectively. The nitrite ion concentration was 3.4- to 9-fold higher in the sheep receiving the molybdenum-supplemented diet.

Figure 6 shows that there was a pronounced rise in ruminal ammonium ion concentration and that the concentration was 1.1- to 1.8-fold higher in sheep receiving the molybdenum-supplemented diet, and of greater importance, the ammonia accumulated in the experimental sheep at a much faster rate during the first seven hours after feeding.

Figure 7 exhibits the blood nitrate concentrations and indicates that the nitrate ions from the sheep fed the basal diet were reaching the blood at a 1.3- to 2.1-fold faster rate than those from the molybdenum-supplemented sheep. It may be seen in Figure 8, however, that the nitrite ion concentration was many times higher in the plasma of the molybdenum-supplemented sheep. Figures 9 and 10 show the coincidental and interrelationship changes in the concentrations of nitrate, nitrite and ammonium nitrogens, in the rumen of the sheep receiving the supplemented and the basal ration, respectively.

DISCUSSION

Trial I. The design of this phase of experiment was suggested by the well-established facts that the nitrate reductase, which catalyses the reduction of nitrate to nitrite in some bacteria, fungi, and green

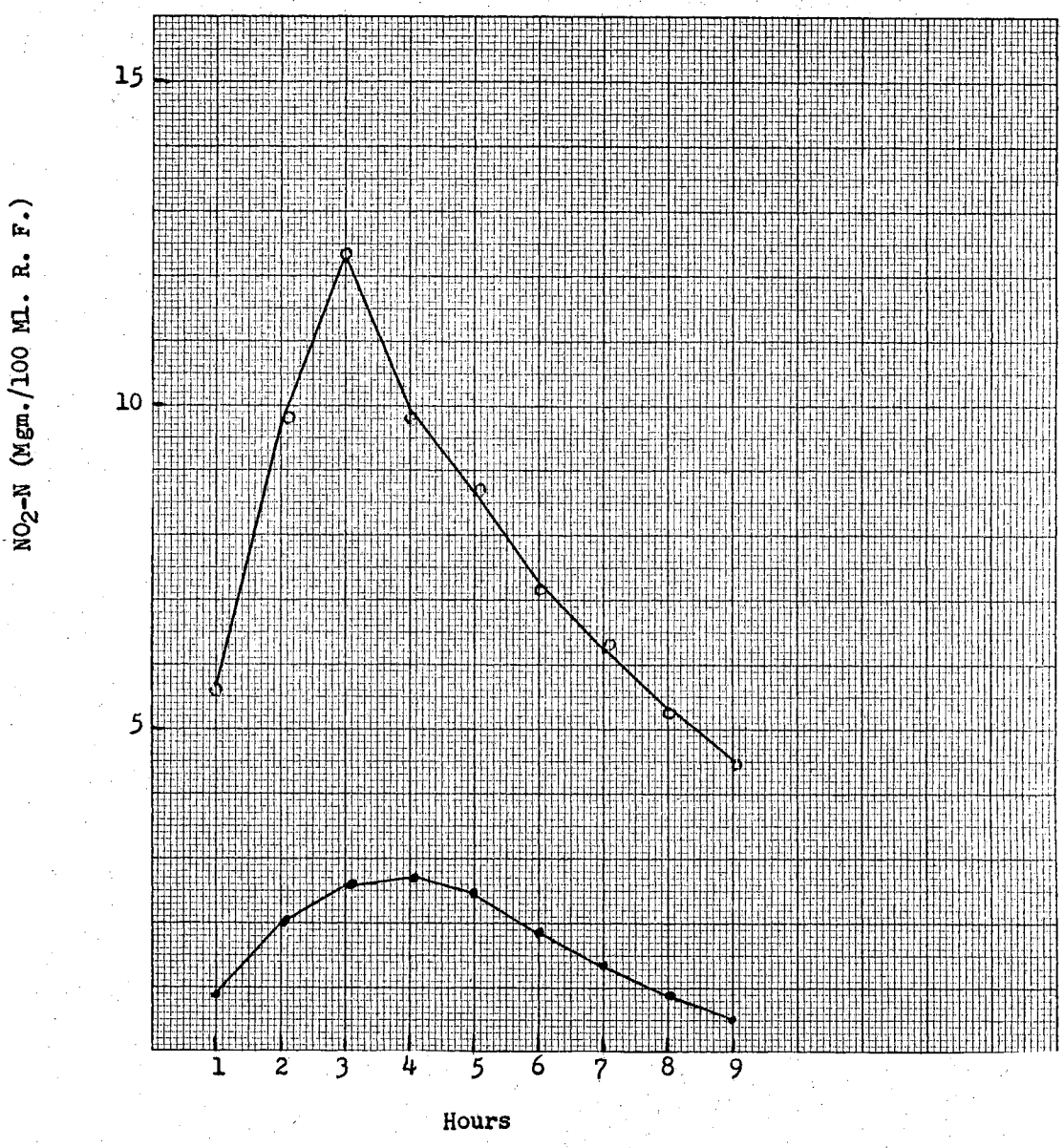


Figure 5. Nitrite-Nitrogen in Rumen of Sheep After Administration of Diets Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

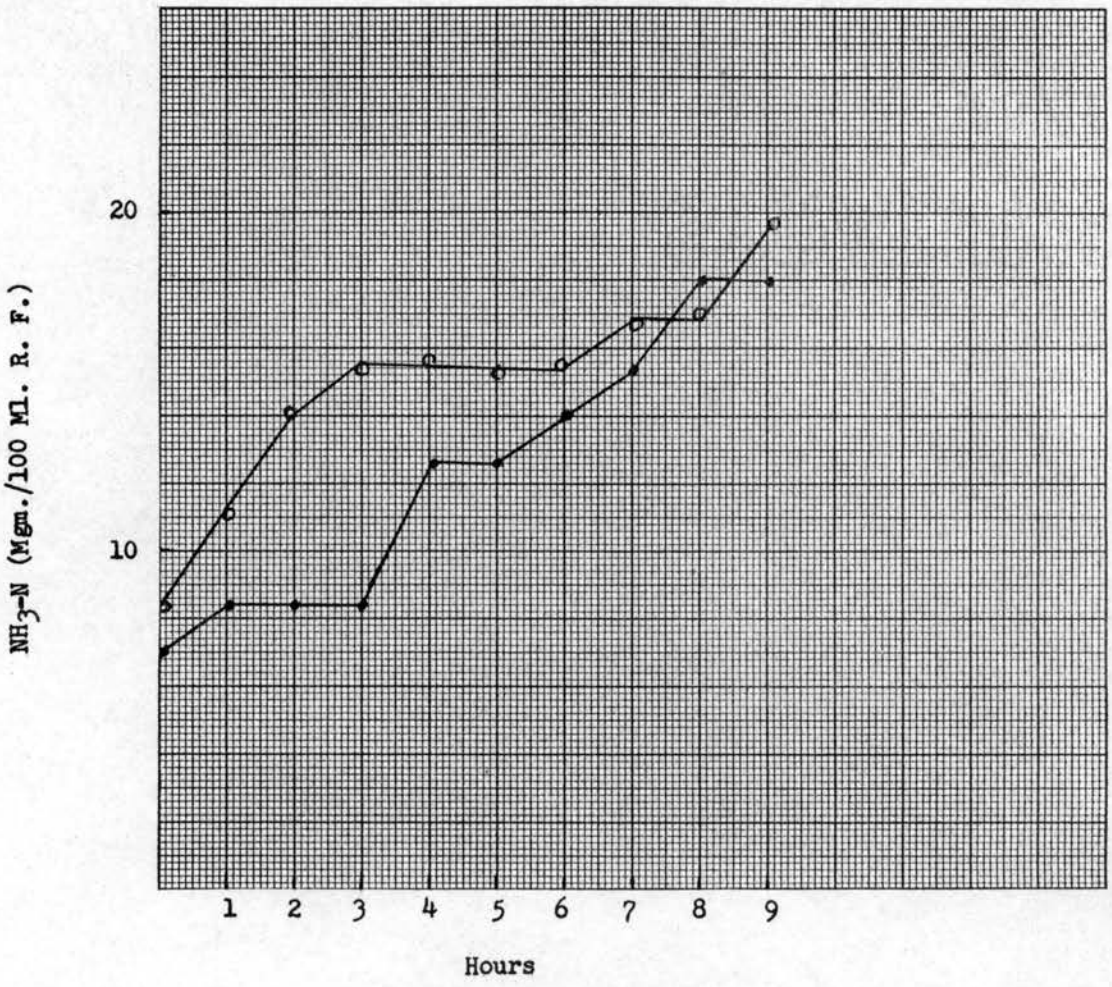


Figure 6. Ammonium-Nitrogen in Rumen of Sheep After Administration of Diets Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

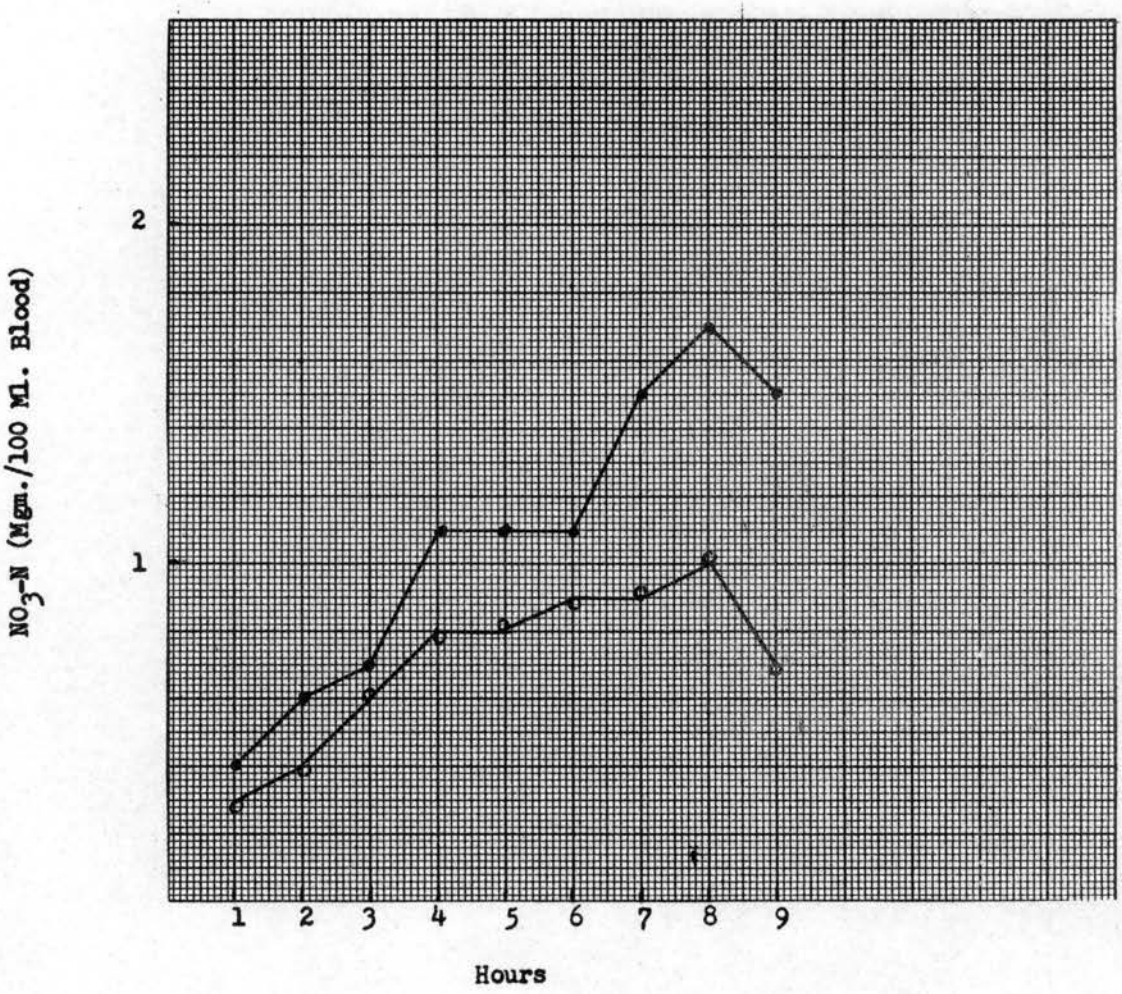


Figure 7. Nitrate-Nitrogen in Blood of Sheep After Administration of Diets Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

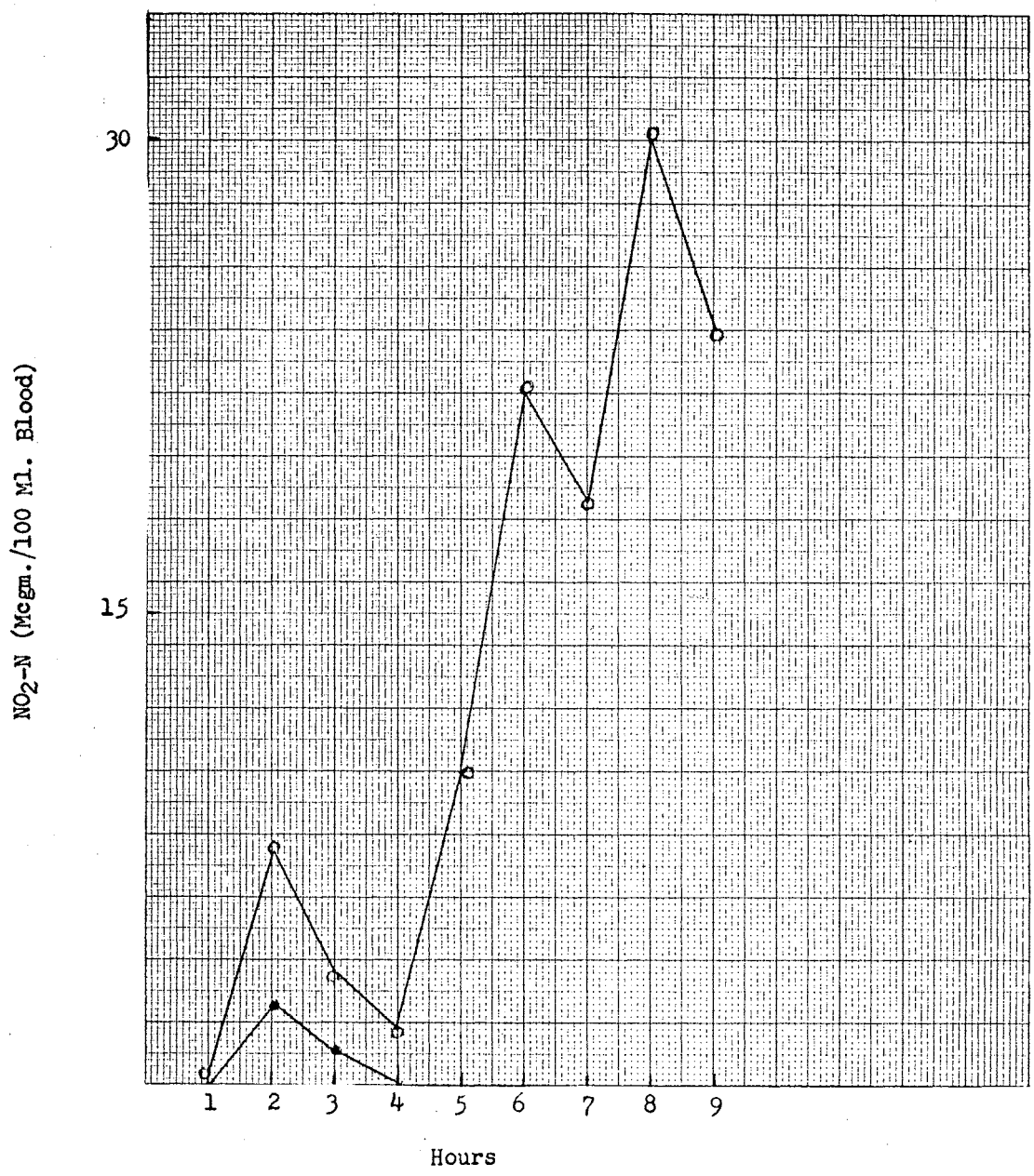


Figure 8. Nitrite-Nitrogen in Blood of Sheep After Administration of Diets Containing Potassium and Sodium Nitrates

● Basal

O Basal Plus Molybdenum

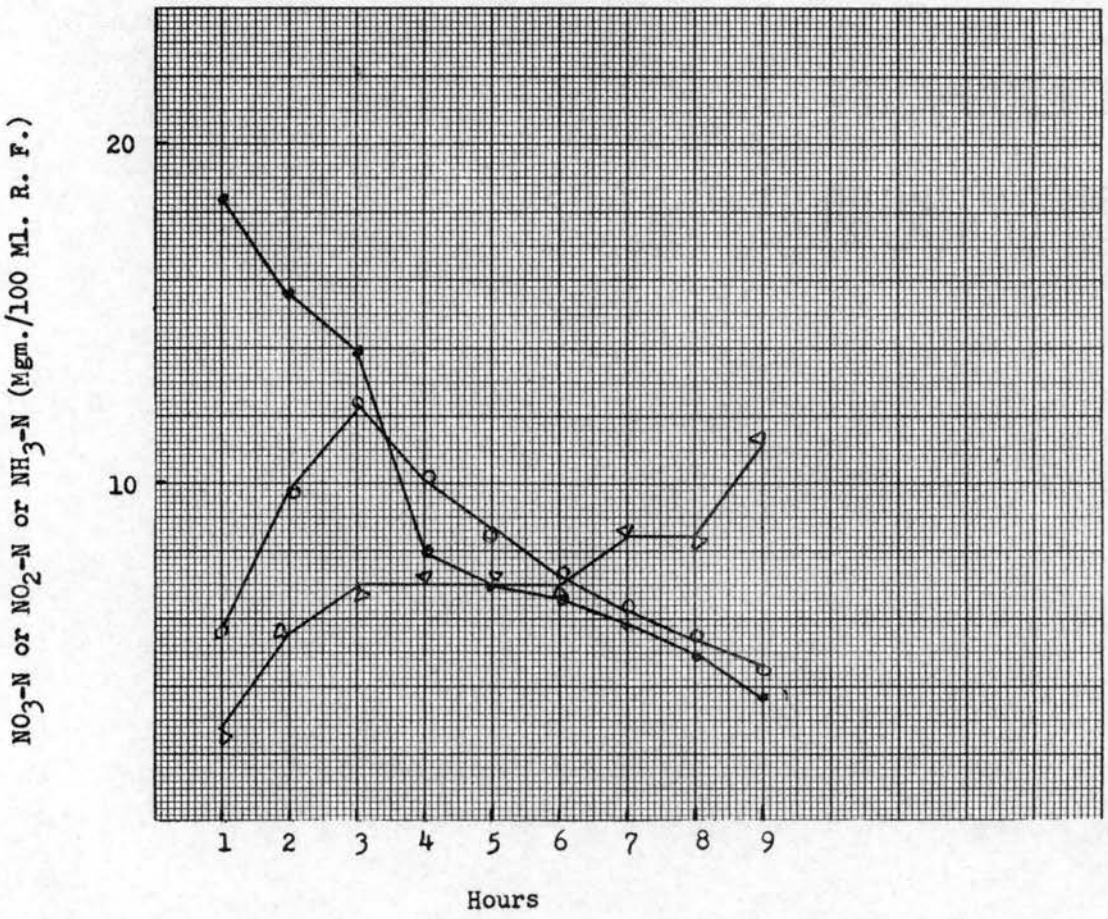


Figure 9. Nitrate Reduction in Rumen of Sheep After Administration of the Molybdenum Supplemented Diet

● Nitrate-Nitrogen

O Nitrite-Nitrogen

△ Ammonium-Nitrogen

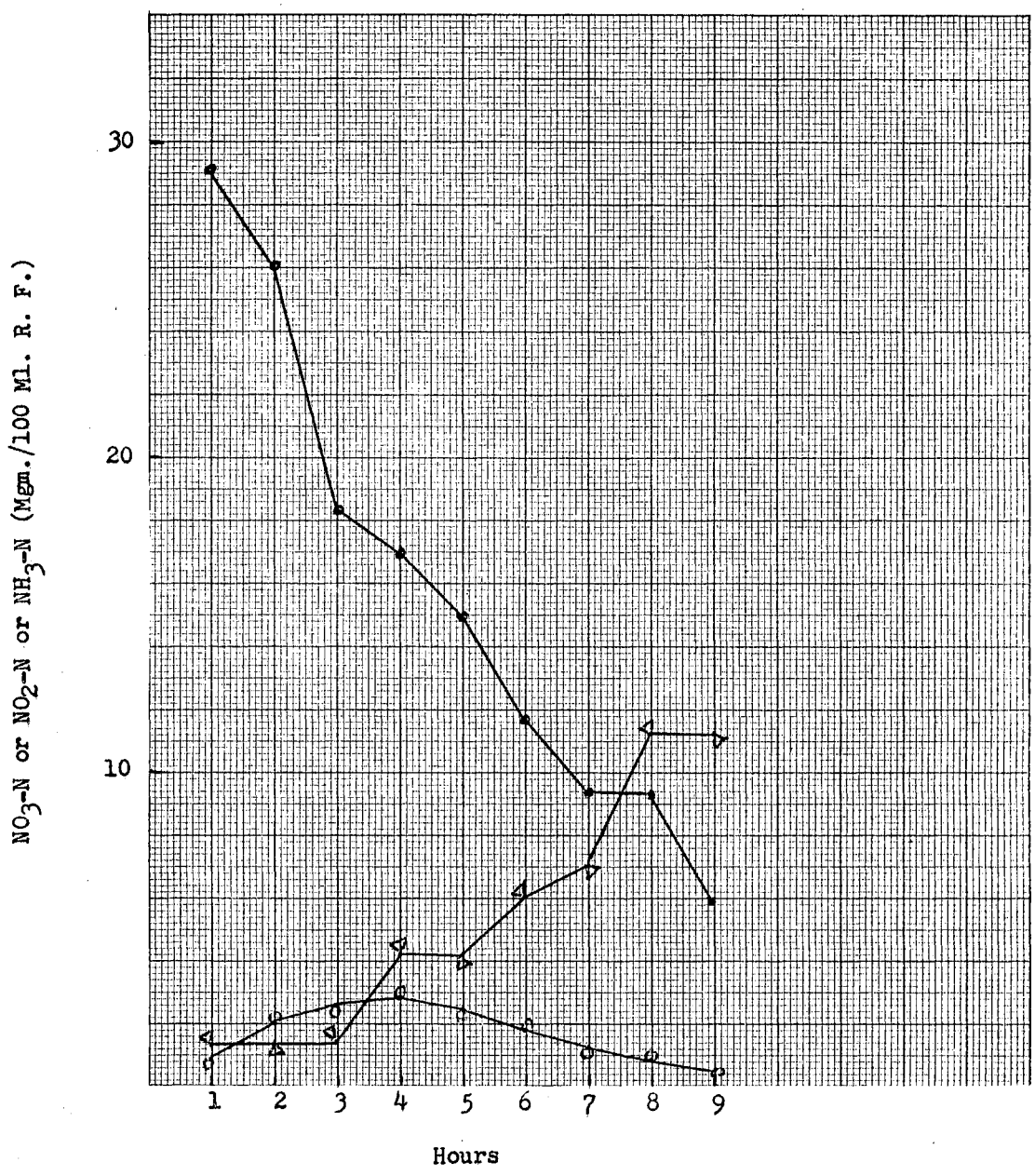


Figure 10. Nitrate Reduction in Rumen of Sheep After Administration of the Basal Diet

● Nitrate-Nitrogen

O Nitrite-Nitrogen

△ Ammonium-Nitrogen

plants is a molybdoflavoprotein (Nicholas et al., 1954a, 1954b, 1954c, 1955a, 1955b; Nicholas, 1959; Nason et al., 1953; Taniguchi et al., 1960a); that iron is required for nitrate reductase activity only in organisms dissimilating nitrate under anaerobic conditions (Etagaki et al., 1959; Taniguchi et al., 1960a; Fewson et al., 1961a, 1961b; Walker et al., 1961a); that both iron and copper are essential for nitrite reductase (Chung et al., 1956; Medina et al., 1957; Nicholas, 1959; Nicholas et al., 1960; Asano, 1960). With this information in mind, the theories were formulated as follows:

(a) The nitrates will be high and, consequently, the nitrites will be low in blood of sheep receiving the basal ration because of the effect of molybdenum deficiency on nitrate reduction to nitrite by rumen microorganisms. Also, tissue xanthine oxidase will be lower in molybdenum-deficient animal tissues. In respect to the formation of nitrite in blood, Westerfeld et al. (1959) found, using in vitro studies, that chicken liver xanthine dehydrogenase reduced sodium molybdate and nitrate, but Pfander et al. (1957) found very little toxicity from nitrate added intravenously. Also, Wang et al. (1961) found no direct evidence for nitrite formation from nitrate in blood when N^{15}-labelled nitrate was added directly into the rumen; thus, it was concluded that the primary site of nitrite formation was in the rumen rather than in the blood. Actually their data supports the idea that the conversion of nitrate into nitrite in the blood was negligible.

(b) The blood of sheep fed the basal ration which was supplemented with 4 ppm molybdenum should be low in nitrates and, consequently, low or high in nitrites depending upon whether the nitrate assimilation

or nitrate dissimilation (nitrate respiration) types, as classified by (Verhoeven, 1956; Sato, 1956a, 1956b; Tuniguchi et al., 1956; Nason, 1958; Fewson et al., 1961c) is predominant in the rumen microorganisms, respectively. It appears that the final products of the first type are nitrogenous cell components; however, the final products which can be found during the second type are as follows: Nitrite, nitric oxide, nitrous oxide, nitrogen, hydroxylamine, or ammonia.

(c) And, in respect to the sheep fed basal ration plus molybdenum and minus both iron and copper, the nitrates will be high in blood if the nitrate dissimilation type (required molybdenum as well as iron) is predominant over the assimilation and, consequently, the nitrites will be low. But the nitrates will be low if nitrate assimilation type, which require molybdenum only, is predominant. The nitrites will be high only because the iron and copper which are essential for nitrite utilization become the limiting factors.

The above theories were based upon results obtained with in vitro trials conducted in closed systems. It is interesting that these in vivo results (Figures 1 and 2) agree so closely with the in vitro results and gives confidence that the in vitro results can be extended to large in vivo units.

The association of highest nitrate levels (Figure 1) in the blood of animals receiving the basal diet with the lowest nitrite levels (Figure 2) during the first 10 intervals of sampling, as compared with levels of nitrate and nitrite of treatment 2 or 3, indicate that molybdenum is the limiting factor for reduction of nitrate to nitrite by rumen microorganisms. The drop in blood nitrates of animals receiving

the basal diet to that level found in the other two treatments during the eleventh and twelfth intervals coincided with the increase in blood nitrites (Figures 1 and 2), and lends further support to the idea of essentiality of molybdenum for reduction of nitrates to nitrites.

The 3.6-fold lower level of blood nitrates accompanied by an 8-fold higher level of blood nitrites in the animals receiving a dietary level of 4 ppm molybdenum indicate that this level of molybdenum induced the rumen microorganisms to reduce rapidly nitrates to nitrites. It is also possible, but doubtful, that the added molybdenum activated xanthine oxidase of the animal tissues and that it might play some role in the conversion of nitrates to nitrites in blood. Sheriha et al. (1962) reported results which indicated that the molybdenum requirement of sheep not receiving nitrates was very low. If the primary site of formation of nitrite is in the rumen, rather than in blood (Pfander et al., 1957; Wang et al., 1961) and the nitrite found in the blood is bound firmly (Wang et al., 1961), it appears paradoxial that nitrites are found in appreciable amounts in blood. In spite of supplementation of the diet with copper and iron, nitrite still accumulated in the rumen and were absorbed into the blood stream; thus, it is also possible that rumen microorganisms reduced nitrates to nitrites more rapidly than in completing the further reduction of the latter. Inhibition of the mechanism responsible for nitrite utilization by the reduction product (Nicholas et al., 1960) and/or the dissimilatory type of nitrate reduction, which is induced by anaerobiosis conditions, could be predominant over assimilatory type in the rumen are possible explanations. Fewson et al. (1961a) found that Neurospora crassa assimilated nitrate under aerobic conditions and that nitrate reductase is molybdenum dependent, but when grown at low

oxygen tension, these microorganism dissimilates nitrate and under these conditions nitrate reductase is dependent upon iron as well as molybdenum. Nitrite under anaerobic conditions accumulated because of the high activity of nitrate reductase.

The blood nitrate and nitrite levels of animals receiving the basal diet plus molybdenum, copper and iron (ration 2) were higher than those of the sheep fed the basal plus only molybdenum and might be explained on the basis of the lower feed intakes of the animals receiving ration 3. The lower feed consumption might have been due to deficiencies of both iron and copper. Such deficiencies could have detrimental effects on both rumen microorganisms and the host which in turn are reflected in reduction of animal appetites. The deficiency of iron in the diet might also reduce the dissimilatory type of nitrate utilization, thus most of the nitrites in blood would have to be formed by the assimilatory type and the unusual association between the accumulation of nitrites with nitrate assimilation might be explained by the copper and iron deficiencies which are essential for nitrite utilization by rumen microorganisms. If such speculation is true, it becomes feasible to conclude that such conditions could cause a nitrogen deficiency for microorganisms and, consequently, to the host. This could explain why there was greater nitrate toxicity per unit of dietary nitrate in the animals receiving the basal diet plus only molybdenum. This idea is supported by the fact that the animals which had previously received the basal plus only molybdenum about doubled their daily intake when copper and iron were added to the diet.

The death of one animal from each treatment 2 and 3 might have been caused by nitrite toxicity or the toxicity of other intermediate products

in the pathway of the nitrate reduction to ammonia, such as nitric oxide, nitrous oxide, and hydroxylamine.

Trial II. Many investigators have reported that nitrate toxicity in ruminants is caused primarily by its reduction products, nitrite and hydroxylamine, which are formed in the rumen. The current studies were designed to go one step further and investigate the role of molybdenum in the reduction of nitrate to nitrite and to follow the absorption of these products into the blood of sheep.

During the preliminary phase of this experiment, and in Trial I, a conditioning period of several days were required before appreciable quantities of nitrite appeared in the rumen fluid after the administration of nitrates into the rumen. A somewhat longer period was required for the nitrites to accumulate in the blood. Wang et al. (1961) also obtained the same results. These observations are interpreted to indicate that time is required, after the introduction of nitrate in the diet, for a change in microflora towards the predominance of those organisms possessing ability to reduce nitrates.

The pH of rumen fluid is one of the very important factors affecting rate of nitrate utilization by rumen microflora. Lewis (1951b) found that washed rumen microorganisms required a pH of 6.5 and 5.6, respectively, for optimum utilization of nitrates and nitrites; the pH optima for the utilization of hydroxylamine was between six and seven. The results of this trial are discussed in terms of the effect of molybdenum as well as changes in rumen pH upon the reduction of nitrate to nitrite and the latter to ammonia.

During the first four hours, the pH of rumen fluid of both sheep dropped from about 7.3 to 5.6, but there were no differences between the

two treatments. During this same period of time, however, the sheep receiving the molybdenum-supplemented diet had a 1.6- to 2.1-fold lower concentration of ruminal nitrate, 3.7- to 6.3-fold higher nitrite and 1.2- to 1.8-fold higher ruminal ammonia level. Blood levels, in time, reflected the ruminal concentrations of these products; the molybdenum-supplemented sheep had a 1.2- to 1.5-fold lower blood nitrate, and a 2.9 to 3.2 higher blood nitrite level than those sheep receiving only the basal diet. These comparisons offer further evidence that rumen microflora, in vivo, require molybdenum for the reduction of nitrate to nitrite, and that body reserves and/or ration contamination did not supply enough of this element for the performance of this reaction. It is interesting to note that the rate at which nitrite was accumulating in the rumen was closely associated with the disappearance of ruminal nitrate and that this occurred when the ruminal pH was favorable for nitrate reduction. When the ruminal pH was favorable for nitrite reduction, there was a slow disappearance of ruminal nitrates and a fast accumulation of ruminal ammonia.

When ruminal pH favored nitrate reduction, nitrite was observed to be rapidly accumulating in the blood. Conversely, when ruminal pH was favorable for nitrite reduction, nitrates were being absorbed into the blood, and in spite of urinary nitrate elimination (Wang et al., 1961) and dilution by the blood, its concentration was increasing with time (Figure 7). The rapidity with which nitrites and nitrate appear in the blood stream support the idea of Wang et al. (1961) that nitrite (and by implication, nitrate and ammonia) is absorbed into the blood from the rumen rather than from the lower sections of the digestive tract.

During the succeeding five hours of sampling, the ruminal pH of

the control sheep remained fairly constant at about 5.6, while that of the sheep receiving the molybdenum-supplemented diet rose steadily to a peak of 6.5 at the seventh hour, and then fell to 6.4 and 6.2 at the eighth and ninth hours, respectively. During this period ruminal nitrate level was 1.3- to 2.1-fold lower in the molybdenum-supplemented sheep. This sheep also had a 3.4- to 9.0-fold higher ruminal nitrite and 1.1- to 1.2-fold higher ammonium ion levels than the control sheep. The blood levels of these products were as expected; the treated sheep had 1.2- to 2.1-fold lower nitrate levels. Nitrite was detected in appreciable quantities in the blood of the molybdenum-supplemented sheep; however, no nitrites were found in the blood of the sheep consuming the molybdenum-deficient diet.

During the last five hours of the test, the ruminal pH of the control sheep was optimum for nitrite reduction, and offers a plausible explanation as to why there was a rapid disappearance of ruminal nitrite, which was accompanied by a rapid accumulation of ruminal ammonium ions. This might be the explanation of why no nitrites were detected in the blood of the control sheep. During this period, however, the nitrate level of the blood of the sheep on the molybdenum-deficient diet increased considerably (Figure 7); these data support the idea of Wang et al. (1961) that the primary site of nitrite formation is in the rumen rather than in the blood. The increased ruminal pH in the treated sheep during the last five hours of the test might be explained on the basis of toxicity to the rumen microflora of some intermediates in the pathway of nitrate reduction to ammonia when accumulated (Verhoeven, 1956; Sato, 1956a, 1956b; Taniguchi et al., 1956, 1960a, 1960b; Heredia et al., 1960; Fewson et al., 1960, 1961a, 1961b, 1961c; Walker et al., 1961a,

1961b, 1961c; Asano, 1959a, 1959b, 1960; Chung et al., 1956; Sacks et al., 1952; Allen et al., 1952; Delwiche, 1959; Zucker et al., 1955; Kono et al., 1960) under anaerobic conditions, at which time nitrate respiration is predominant over nitrate assimilation. Hydroxylamine, which is known to be highly toxic to almost all living organisms, has been shown (Jamieson, 1958) to be present in the rumen of normal sheep on pasture and in much higher concentrations in ruminal fluid of animals metabolizing added nitrite.

During the last five hours of the experiment, the ruminal pH of the treated animal shifted from one that, at the beginning was favorable for nitrite reduction to one that was favorable for nitrate reduction. This offers explanations for the slow disappearance of ruminal nitrites which was accompanied by a decreasing rate of ruminal ammonia production and a rapid accumulation of nitrites in the blood. Ruminal nitrite disappearance, which proceeded at a decreasing rate during the last five hours of the trial, might also be due to the inhibition of rumen microflora of reduction products; Nicholas et al. (1960) have shown that nitrite reduction products inhibit nitrite reductase in Neurospora crassa and that the addition of hyponitrite but not hydroxylamine depressed the activity of the enzyme.

The interrelationship changes in ruminal concentrations of nitrate, nitrite and ammonia are shown for the experimental and control sheep, respectively, in Figures 9 and 10 and summarizes the information shown in Figures 4, 5 and 6. It is interesting to note that at the ninth hour, the concentration of ammonium nitrogen was higher than that of the nitrate plus nitrite nitrogen. Wang et al. (1961) also noted this in his experimental animals and suggested that ruminal conditions at that time

were more favorable for the absorption of nitrate and nitrite than for the absorption of ammonia. The toxicity of hydroxylamine to micro-organisms as reported by Borex et al., 1951; Krishnaswang et al., 1956; Saxena et al., 1957; Moyed et al., 1957; and Webster, 1957, must also be considered as a possible explanation.

GENERAL DISCUSSION

The idea behind the first phase (Trial I) of this experiment, was to provide information concerning the reduction of nitrate to nitrite in vivo in the rumen of sheep by using the appearance of nitrate and nitrite in blood as criteria. The main interest in these studies was on evaluation of the molybdenum role in this reduction. The results of this trial supported the idea of the molybdenum essentiality for nitrate reduction to nitrite, but failed to indicate whether the rumen or the blood of sheep is the primary site for this reduction. The variations in feed consumption made the results of this trial difficult for interpretation; therefore, the design of Trial II was dictated by a desire to answer the questions which were raised and left open by Trial I. Since the studies of this phase of experiment followed periodically, after the administration of the diet with and without added molybdenum via a fistula, the appearance rates of nitrate and nitrite in the blood stream of sheep coincided with the changes in nitrate, nitrite ammonium ion concentration and pH readings of the rumen.

The results of the two trials indicate strongly that molybdenum is required by rumen microflora for the reduction of nitrate to nitrite and that ruminal pH plays a very important role in the rates with which nitrate, nitrite and possibly other nitrate-reduction products are

utilized by rumen microflora. These results also indicate that the actions of rumen microflora in nitrate reduction can be predicted from the results of *in vitro* bacterial studies.

SUMMARY

Two trials were conducted with sheep to study nitrate reduction. In the first trial sheep were fed a purified diet in which nitrates were the sole source of dietary nitrogen. Molybdenum was omitted from ration 1 and added to rations 2 and 3. Copper and iron were added to rations 1 and 2 and omitted from ration 3. When molybdenum was omitted, there was an accumulation of nitrates in the blood of the sheep, but when molybdenum was present in the diet, there was an accumulation of nitrites irregardless of whether dietary copper and iron were present. It is suggested that under the anaerobic conditions in the rumen, nitrate dissimilation by the rumen microflora is predominant over nitrate assimilation. Feed consumption was lower in sheep receiving the diet containing no added iron or copper.

In the second trial, a purified diet with and without added molybdenum, was also used, but was administered via rumen fistula to sheep. Rumen samples as well as blood from the jugular vein were taken at hourly intervals for nine hours after administration. Molybdenum was required by the rumen microflora for reduction of nitrate to nitrite and ruminal pH seemed to play a predominant role in influencing the rates with which nitrates and nitrites were utilized by the rumen microflora.

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EXPERIMENT II

THE TOXICITY OF HYDROXYLAMINE

REVIEW OF LITERATURE

Hydroxylamine Toxicity

Chanutin et al. (1954) reported that a single intravenous injection in rats of 0.14 mM/kgm. hydroxylamine hydrochloride caused a slight increase in serum iron concentration without an appreciable change in total hemoglobin concentrations or reticulocyte counts. At a higher level (0.28 mM/kgm. hydroxylamine) hyperferremia was manifested during the first 14 days and on the fifteenth day the serum iron concentration was markedly decreased and remained below the control level during the period of observation. During the second week the hemoglobin concentration decreased but subsequently increased. The reticulocyte count was greatest (10 percent) during the third week when the hemoglobin concentration begins to return to the control range.

Administration of hydroxylamine alone (0.56 mM/kgm.) caused a slight decrease in serum iron and hemoglobin concentrations and some increase in the reticulocyte count. After treatment with hydroxylamine plus irradiation, a marked hyperferremia was noted in all groups during the first nine days and on the tenth and twelfth days the serum iron concentrations varied over a wide range and included many values within the control limits, subsequently a hypoferremia was observed in most of the animals between the fourteenth and twenty-fourth days. The hemoglobin concentration decreased gradually after treatment and reached minimum values between the eighth and twelfth days. Increased reticulocyte

counts are seen during the period of anemia, the decrease in the number of these cells can be correlated with the return of hemoglobin concentration towards the control level.

Jamieson (1958) found that hydroxylamine in small amounts caused a severe hemolytic anemia in sheep. Oral doses of 40 to 80 mgm./kgm., and intravenous doses of 1 to 7 mgm./kgm. body weight of hydroxylamine hydrochloride brought about a lowering of the hematocrit readings within 12 to 15 days from the normal range of 30 to 40 percent to values as low as 10 to 20 percent. A highly significant decrease in growth of hydroxylamine-treated animals relative to controls grazing the same pasture was also noted.

Borek et al. (1951) found that hydroxylamine inhibited the growth of Lactobacillus arabinosus and Proteus vulgaris. The effect of minimal inhibiting doses of the anime can be reversed by ammonia or glutamine and to a lesser extent by asparagine in L. arobinosus and by asparagine, ammonia, and glutamine in P. vulgaris. Since hydroxylamine combines readily with carbonyl groups, it has herefore been assumed that its growth-inhibiting action was caused by interference with the utilization of metabolites containing such groupings. It is suggested that hydroxylamine in low concentration interferes with the utilization of glutamine, asparagine and ammonia; in high concentrations it probably blocks other metabolic pathways as well.

Lipmann et al. (1945) found that hydroxamic acid, which is formed by a reaction between hydroxylamine and acyl groups, is capable of forming a stable trivalent iron complex. Wainfan et al. (1957) found that hydroxylamine will selectively react with thioacyls and/or with carboxylic amhydrides.

Wald et al. (1950) found that cattle or frog rhodopsin, bleached in solution in the presence of high concentrations of synthetic retinene, regenerated about 70 percent of the original content of rhodopsin when placed in the dark. It is inhibited, competitively, by formaldehyde, and also with such retinene-trapping reagents as hydroxylamine.

Wald et al. (1955) found that hydroxylamine bleaches iodopsin, yet does not poison its synthesis. Hydroxylamine acts by competing with the opsins for retinene. It competes successfully with chicken, cattle, or frog scotopsin, thus blocks rhodopsin synthesis. It appears to be less efficient than photopsin in trapping retinene, but does not block iodopsin synthesis.

Adams et al. (1958) found that hydroxylamine is capable of removing retinene from the protein opsin after the retinene has been isomerized to a sterically incompatible form. Dietrich et al. (1956) reported that hydroxylamine was observed to inhibit xanthine oxidase activity competitively. Krishnaswamy et al. (1956) reported that glutamic acid decarboxylase has been detected in yeast, R. glutinis. Pyridoxal phosphate could restore the activity of dialysed preparations and reverse inhibition caused by hydroxylamine. Hagihara et al. (1956) found that KCN, NaN_3, and NH_2OH completely inactivated crystalline beef liver catalase.

Saxena et al. (1957), in studying transamination reactions in P. pestis, used α-Ketoglularate (α-KG), pyruvate and oxalacetate as acceptors and a number of amino donors and found that cell-free extracts of this organism possess enzymes which transfer amino-groups to α-KG from a number of donors (L. aspartate, L. asparagine, L. alanine, L. leucine, L. isoleucine, L. phenylalanine, L. methionine, L. valine and L. tyrosine). An extraneous supply of pyridoxal phosphate was found essential for

transferring of $-NH_2$ group from alanine, but was unnecessary when aspartate, leucine or phenylalanine were the donors. Hydrazine sulfate, iodoacetate, hydroxylamine-HCL, and $CuSO_4$ strongly inhibited these reactions.

Moyed et al. (1957) reported that an enzyme catalyzing the amination of xanthosine-5'-phosphate by ammonia to produce guanosine 5'-phosphate was found in extracts of A. aerogenes. The irreversible conversion requires magnesium and adenosine triphosphate (ATP), which is split concomitantly into adenosine 5'-phosphate and pyrophosphate. The reaction is inhibited by hydroxylamine, which appears to inactivate the enzyme when ATP and xanthosine 5'-phosphate are present. Xanthosine 5'-phosphate aminase is essential for the biosynthesis of nucleic acid guanine.

Webster (1957a) found that ribonucleoprotein particles from both yeast and pea seedlings are able to incorporate amino acids into protein. In addition, the pea seedling particles, if prepared in the absence of salts, catalyze amino acid-promoted pyrophosphate-adenosine triphosphate (ATP) and adenosine monophosphate (AMP) - ATP exchanges, amino acid hydroxamate formation, and the incorporation of adenosine 5'-phosphate into ribonucleic acid. Amino acid incorporation and the amino acid-promoted AMP-ATP exchange are inhibited by ribonuclease, chloramphenicol, hydroxylamine and rubidium.

Webster (1957b) found that ribonucleoprotein particles isolated from pea seedlings incorporated the carbon of carbon dioxide, formate, glycine, adenine, and uracil, as well as orthophosphate into ribonucleic acid; the incorporation required the presence of mitochondrial preparation, soluble cytoplasmic protein, and magnesium. The system also catalyzes the incorporation of the above precursors into soluble nucleotides. Incorporation

of precursors into polynucleotide is inhibited by various inhibitors of protein synthesis (amino acid analogs, hydroxylamine, and chloramphenicol). It is concluded that ribonucleic acid synthesis by ribonucleoprotein particles is normally linked to concurrent protein synthesis, or the two synthesis share a common intermediate.

Altman et al. (1947) found that aqueous extracts of the acetone powder of pigeon liver contained an enzyme system capable of synthesizing triphosphopyridine nucleotide (TPN) when ATP was added. Maximal synthesis was obtained with a system containing nicotinamide, ATP and ribose. All the nicotinamide originally present was accounted for by the TPN synthesized. The synthesis was inhibited by 10^{-4}M NaCN and by 10^{-3}M NH_2OH.

Verhoeven (1952) detected hydroxylamine in culture medium during dissimilatory nitrate reduction by Denitrobacillus licheniformis. Also, reduction of hydroxylamine to ammonia has been demonstrated in a number of microorganisms (Silver et al., 1954; Spencer et al., 1957; Medina et al., 1957; Fewson et al., 1961; Zucker et al., 1955; Kono et al., 1960; Walker et al., 1961). Lewis (1951a, 1951b) has shown that both nitrate and nitrite are ultimately reduced to ammonia and that hydroxylamine might be an intermediate in the reduction sequence; this compound can be reduced by washed suspensions of sheep rumen microorganisms to ammonia. Jamieson (1958) detected hydroxylamine in the rumen of sheep on pasture and at higher concentrations in the rumen of sheep metabolizing added nitrite. The present investigation was designed to provide information concerning the effects of hydroxylamine on (a) liver carotenoid and vitamin A levels, (b) plasma vitamin A levels, (c) hemoglobin level, (d) growth rate, and (e) feed consumption in sheep.

EXPERIMENTAL PROCEDURE

Six ewe lambs averaging 53 pounds were divided on the basis of weight into two equal groups of three. Each group was kept indoors and group-fed the following pelleted ration, in percent: Ground milo, 60.0; alfalfa meal, 10.0; cottonseed meal, 15.0; cottonseed hulls, 13.5; sodium chloride, 0.5; dicalcium phosphate, 0.5; calcium carbonate 0.05; and santoquin, 0.025. In addition the diet contained supplemental vitamin A and D at the levels of 1,000 and 250 I.U. of vitamin A and D per lb., respectively. The animal groups were kept in separate pens during a 12-day period and were group-fed twice daily and water was available to the animals at all times. During the first 10 days the control sheep received a daily intravenous injection of four ml. of physiological saline solution, while at the same time, the treated animals received eight mgm./kgm. body weight of hydroxylamine-hydrochloride, which was dissolved in four ml. of physiological saline solution; the hydroxylamine was mixed with the saline daily just prior to injection.

Blood samples were removed daily, one hour after the injection of the animals, for hemoglobin determination. The AO Spencer Hb-Meter was used for hemoglobin determination. Fifty ml. of blood were taken on zero, eight and twelve days after injections were analyzed for carotene and vitamin A. The blood was centrifuged within an hour and the plasma was removed, flushed with CO_2 and frozen until analyzed. At the end of the experiment, all animals were sacrificed and liver samples obtained for analyses of carotenoid and vitamin A contents. Analysis for carotenes were conducted by the methods of A.O.A.C. (1960) while the analysis for vitamin A was conducted by the method which has been described by Carr

and Price (1926). Statistical analyses of the data were conducted by the Student's "t" Test as described by Snedecor (1956).

RESULTS

The effects of hydroxylamine upon levels of weight gains, feed consumption, liver carotenoids, liver vitamin A, and plasma vitamin A are shown in Table I. Hydroxylamine reduced both feed consumption and gains ($P<0.01$). The liver values are reported on the basis of fresh liver and it may be seen that neither liver carotenoids nor liver vitamin A were significantly ($P>0.05$) changed by hydroxylamine administration. Plasma vitamin A values were reduced by the eighth day ($P<0.05$) and still further ($P<0.01$) by the twelfth day after hydroxylamine administration was initiated.

Hydroxylamine reduced hemoglobin levels and these results are shown in Figure 1. It is interesting to note that differences between the two groups were significant ($P<0.01$) four days after the experiment was initiated and that the differences become greater as the experiment continued.

DISCUSSION

It is generally recognized that nitrate toxicity for ruminants is caused primarily by its reduction products, such as nitrite and hydroxylamine, which accumulate in the rumen under certain conditions and pass into the blood stream in appreciable quantities. The current studies, while re-emphasizing the effects of hydroxylamine in causing hemolytic anemia and growth inhibition in sheep, go one step further and investigate the effect of this compound upon liver carotenoids, liver

TABLE I

EFFECT OF DAILY INTRAVENOUS ADMINISTRATION OF HYDROXYLAMINE-HCL (8 MGM./KGM. BODY WEIGHT) ON WEIGHT GAINS, FEED CONSUMPTION, LIVER CAROTENOIDS, LIVER VITAMIN A AND BLOOD PLASMA VITAMIN A

Mean	Group	
	Control	Treated
Daily Gain	0.54 ± 0.19[b]	-0.17** ± 0.02
Daily Feed Consumption	2.20	1.40
Plasma Vitamin A (Mcgm./100 Ml.)[a]		
Days After Initial Injection		
0	31.7 ± 2.8	31.3 ± 0.7
8	44.0 ± 7.6	17.7* ± 2.2
12	50.3 ± 3.0	25.3** ± 2.4
Liver Vitamin A, Mcgm./Gm.	66.7 ± 6.8	52.0[c] ± 7.2
Liver Carotenoids, Mgm./Gm.	36.7 ± 1.7	28.3 ± 7.4

[a]Vitamin A and carotenoid analysis were determined by Dr. G. S. Smith, Department of Animal Science, University of Illinois, Urbana, Illinois.

[b]Standard error.

[c]($P < 0.20$)

*($P < 0.05$)

**($P < 0.01$)

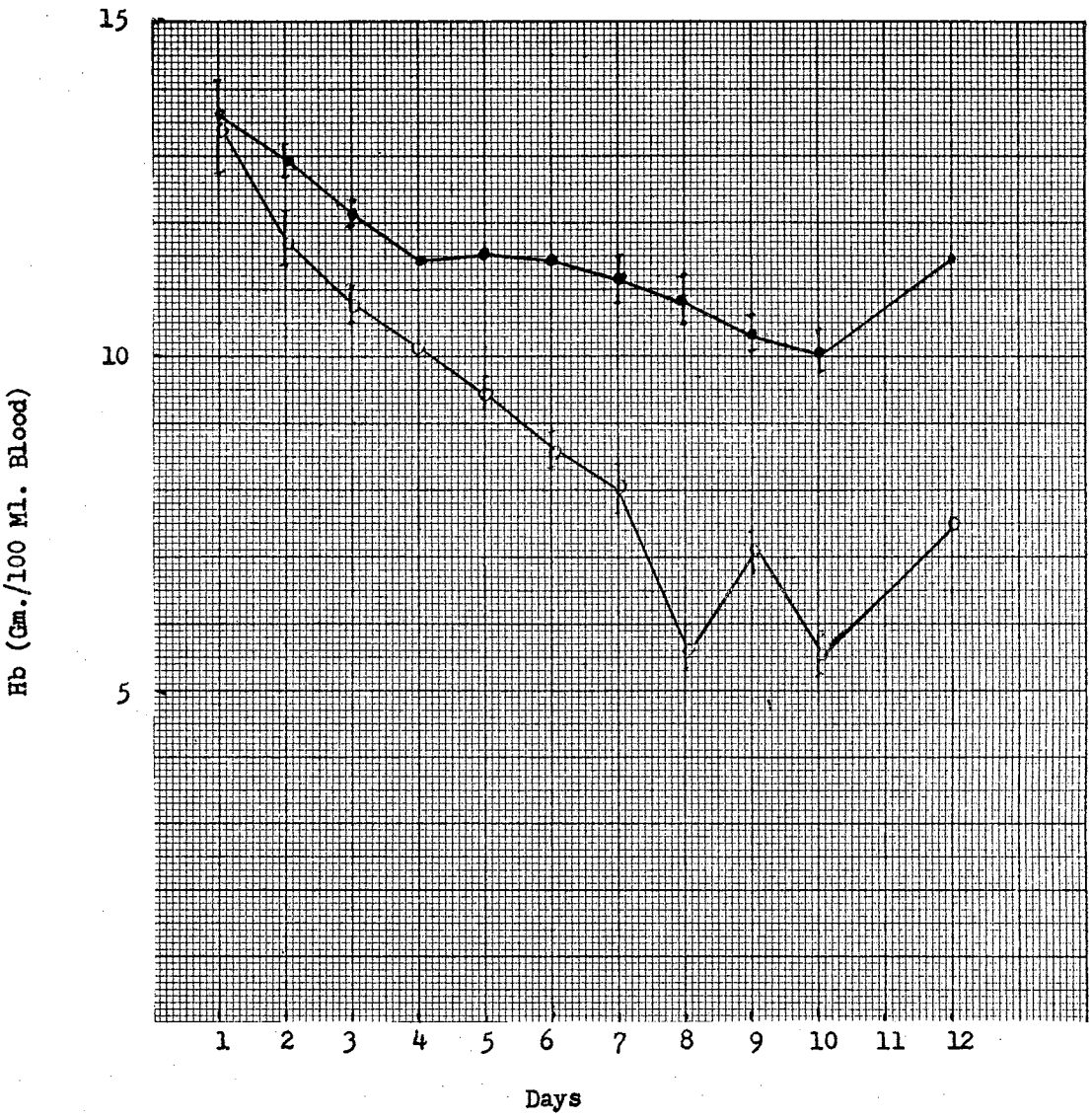

Figure 1. Effect of Intravenous Administration of Hydroxylamine-HCL (8 Mgm./Kgm. Body Weight) Upon Sheep Hemoglobin Levels

● Control Group

O Experimental Group

I Standard Error

vitamin A and plasma vitamin A levels in sheep. These investigations were suggested by the properties of hydroxylamine which allow it to combine readily with the carbonyl groups and interfere with the utilization of metabolites containing such groupings. This idea was supported by the in vitro results of Wald et al. (1950, 1955) in which they found that hydroxylamine competes successfully with chicken, cattle or frog scotopsin thereby blocking rhodopsin synthesis. Adams et al. (1958) found hydroxylamine is capable of removing retinene from the protein opsin after the retinene has been isomerized to a sterically incompatible form.

It can be noted (Table I) that the control sheep were able to increase plasma vitamin A levels from about 32 to 50 mcgm./100 ml. plasma during the 12-day test. These animals, just previous to the initiation of the experiment, had been nursing on their mothers and receiving a supplemental creep ration, which contained neither vitamin A nor carotene. The control diet used in this trial contained 1,040 I.U. of preformed vitamin A and 1.74 mgm. (980 I.U.) of carotenoid pigments per pound of diet; thus, the results are not surprising. The treated sheep showed a decrease in plasma level of vitamin A; however, it must be pointed out that hydroxylamine administration affected appetite and, for this reason, the treated sheep were consuming less vitamin per day than their controls. Actually for the total experiment each sheep receiving the control diet consumed 24.2 pounds of feed which supplied 48,884 I.U. of vitamin A activity (25,168 I.U. vitamin A and 23,716 I.U. vitamin A from carotene) while each sheep getting the injections of hydroxylamine only consumed 15.4 pounds of feed, which supplied 21,108 I.U. vitamin A activity (16,016 I.U. of vitamin A and 15,092 I.U. of vitamin A activity

from carotene). If the requirement for vitamin A be on the basis of body weight (NRC, 1957) it can be calculated that if an intake of 48,884 I.U. caused an increase of 18.6 mcgm./100 ml. plasma in the control sheep, an intake of 31,108 I.U. should have caused an increase of 11.8 mcgm./100 ml. plasma; instead, there was an actual decrease from 31.3 mcgm./100 ml. of plasma to 25.3 mcgm./100 ml. plasma. This calculation does serve to indicate that injection of hydroxylamine did increase destruction of plasma vitamin A and that a decrease in feed consumption was not the cause for the decrease noted in this trial. These data conform with the results of Wald et al. (1950, 1955) and Adams et al. (1958). It is also of interest to note that the plasma vitamin A levels were lower on the eighth day after injections were started than on the twelfth day. It is possible that the gains in plasma vitamin A level were made after injections ceased on the tenth day. Unfortunately, the animals were not bled on the tenth day; therefore, no information on this point is available.

The control sheep gained very well during this short experiment, while the treated sheep lost weight. The treated animals apparently were in stress for several hours after hydroxylamine was injected. Jamieson (1958) also noted a great decrease in growth of sheep receiving hydroxylamine while on pasture. It was also reported by Webster (1957a, 1957b) that the ability of nucleo-protein from yeast and pea seedlings to incorporate amino acids to protein was greatly inhibited by the presence of hydroxylamine.

The daily injection of hydroxylamine caused severe hemolytic anemia (Figure 1) during the 10-day injection period. The effect was very rapid and by the end of the third day differences between the control and treated

animals were significant ($P < 0.01$). Actually, as can be seen in Figure 1, the differences between treatments became apparent before the fourth day. Jamieson (1958) noted that hydroxylamine lowered hematocrit readings from 30-40 to 10-20 percent within 12 to 15 days.

Differences between the control and treated animals were not significant ($P > 0.05$) when liver carotenoids and vitamin A were considered; however, it will be noted that the treated animals apparently had a lower level of vitamin A ($P < 0.2$). It seems reasonable to assume that if the experiment had been continued longer that the lowered level of plasma vitamin A in the treated animals would have resulted in lower liver vitamin A values in these animals.

SUMMARY

Intravenous administration of hydroxylamine-hydrochloride at a level of 8.0 mgm./kgm. body weight reduced feed consumptions, weight gains, vitamin A level, and hemoglobin level in sheep.

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EXPERIMENT III

THE INTERRELATIONS OF COPPER, SULFATE SULFUR AND MOLYBDENUM IN NUTRITION

REVIEW OF LITERATURE

Molybdenum, Copper and Sulfur Interactions

Molybdenum Toxicity. Wynne et al. (1956) found that sheep fed a basal diet containing 5.2 ppm of copper, 0.8 ppm of molybdenum and 0.04 percent inorganic sulfate maintained normal liver and blood copper concentrations. When fed the same diet supplemented to provide 5.1 ppm molybdenum and 0.40 percent sulfate, there was a progressive fall in liver and blood copper levels, and dystrophic wool and hypochromotrichia were observed. When only sulfate was added to the basal diet, liver copper levels fell but not to levels associated with hypocupremia. When only molybdenum was added, the copper level was depressed moderately.

Mylrea (1958) found that the mean liver copper concentration increased in steers fed a basal diet containing 7.6 ppm copper, 2.4 ppm molybdenum, 91 ppm manganese, and 0.03 percent sulfate. Steers fed a similar diet containing 0.55 percent sulfate, with or without an increase in the manganese content to 391 ppm, had a highly significant reduction in liver copper levels. Serum inorganic sulfate levels also increased. When the molybdenum content was increased to 9.2 ppm, there was a further reduction in the liver and blood copper concentrations in steers fed high sulfate diets. There was no definite effect on those receiving the low sulfate diet. Manganese again appeared to be without effect. Even though the very low liver and blood copper levels were attained and maintained for 15 weeks, there was no clinical evidence of hypocuprosis.

Cunningham et al. (1959) fed cattle a ration low in copper and molybdenum, and which contained 0.1 percent of inorganic sulfate. Additional molybdenum increased blood and liver molybdenum and decreased blood and liver copper, while additional sulfate and molybdenum prevented the accumulation of molybdenum in blood or liver, but did not increase the loss of copper from blood or liver. They concluded that 0.1 percent inorganic sulfate in the basal diet provided for any possible interaction between molybdenum and sulfate.

Feaster et al. (1959) reported that rabbits fed 0.15 percent of molybdenum in the diet for five weeks developed molybdenum toxicity symptoms and when S^{35} was given orally the levels of S^{35} found in the livers and bones of the rabbits receiving molybdenum were lower than in their controls. Urinary excretion of S^{35} was higher, indicating decreased retention of sulfur in the animals on high molybdenum diets.

Miller et al. (1956) found that dietary inorganic sulfate had an alleviating effect on the molybdenum-induced rat growth inhibition. The inclusion of molybdenum in the diet caused an increase in the liver and blood levels of molybdenum and copper. This increase was reduced when sulfate was added to the molybdenum-containing ration. The growth inhibition caused by the 75 ppm molybdenum could be overcome by the addition of 2,200 ppm sulfate. When 100 ppm molybdenum was added to the diet, it appeared that a level of sulfate between 800 and 2,200 ppm exerted its maximum growth-protective effect.

Reen (1959) studying the specificity of molybdenum-sulfate interrelationship in rats found that sodium sulfate alleviated the effect of molybdenum toxicity, whereas sodium citrate, tartrate, acetate, bromide, chloride and nitrate did not. Potassium thiomolybdate produced an

extreme toxicosis presumably through the release of hydrogen sulfide which is believed to potentiate molybdenum toxicity. This condition was not ameliorated by sodium sulfate and they found that levels as low as 5 mgm. of molybdenum per 100 gm. of diet resulted in reduced growth of weanling rats when the sulfate level of the diet was low.

Miller et al. (1958) reported a slight reduction in the chick rate of growth was noted in diets containing 500 ppm molybdenum and increasing the molybdenum content of the diet to 2,250 ppm resulted in a very slow rate of growth. The addition of inorganic sulfate to the diet apparently alleviated a part of molybdenum-induced growth inhibition. The level of molybdenum in the blood and the liver of chicks fed the high molybdenum diet was reduced by increasing of the intake of sulfate.

Miller et al. (1959) showed that the addition of molybdenum plus thiosulfate will cause feather depigmentation in growing chicks. Supplementation of the diet with vitamins or lysine did not prevent feather depigmentation when the diet contained molybdenum and thiosulfate. No feather depigmentation occured if 50 ppm of copper was added to the diet. Chicks having feather depigmentation had higher copper, molybdenum and S^{35} content in liver tissues than the basal group, or those receiving supplementary thiosulfate, or molybdenum. In all groups fed radioactive sodium thiosulfate, radioactivity was found in taurine fraction but not in methionine or cystine fraction of liver hydrolysate.

Smith et al. (1946) found that the feeding of excess zinc to young rats induced a microcytic and hypochromic anemia. Additional dietary copper increased the hemoglobin level, while a mixture of iron, copper and cobalt essentially maintained hemoglobin at normal levels; iron or cobalt supplements alone had no affect.

Gray et al. (1950) using the milk powder diet, studied the interrelationships of copper, molybdenum, zinc, and lead in the nutrition of the rat. The effect of molybdenum in retarding growth and of zinc in producing anemia were studied. The ability of the copper to correct the effect of molybdenum on growth was not clearly defined at the mineral levels used; however, copper corrected the anemia caused by zinc. Additional zinc did not retard the growth of rats, but additional molybdenum and zinc in the diet resulted in significantly poorer growth than caused by the addition of only molybdenum.

Dick (1956) found that the addition of molybdenum and manganese exerted a severely limiting effect on copper retention when sheep consumed a high protein diet. Dick (1954) found that increased copper intake of sheep is reflected by increased copper content of the liver; the amount of the copper accumulated by the animal appears to be proportional to the copper intake within the range of 3 to 20 mgm. per day. Liver copper storage over a period of six months was found to be 4.5 to 5 percent of the intake. The addition of dietary ferrous sulfide lowered the expected copper accumulation in the liver by 75 percent. Molybdenum had a severely limiting effect on the liver copper level and this effect was only observed when the diet also contained a sufficient quantity of inorganic sulfate; the limitation on copper storage was proportional to the sulfate content of the diet.

Mills et al. (1958) fed rats a diet containing 800 ppm of molybdenum and found a 36 percent depression of growth during a five week test. The inclusion of 0.29 percent of sulfate in the molybdenum supplemented diet largely prevented the decline in growth. A marked depression of sulfide oxidase activity was noted in homogenate of livers from rats suffering

from molybdenum toxicosis. Alkaline phosphatase/DNA and alkaline phosphatase/RNA ratios were determined on a normal and "high molybdenum" rat liver and kidney and it was found that treatments caused marked differences in these ratios. There was an elevation of liver alkaline phosphatase and depression of kidney alkaline phosphatase activity. Molybdenum-toxicity apparently had no effect on activity of liver cystine desulfhydrase.

Halverson et al. (1960) found that an excessive level of dietary molybdenum produced a profound depression on growth but that there was a low incidence of anemia and diarrhea in rats fed a diet low in copper. Supplementing the diet with copper prevented anemia and diarrhea, but did not restore growth. Administration of an excessive dietary level of cystine to rats fed the high-molybdenum--low-copper diet caused anemia, diarrhea and some fatalities. The toxic effects of cystine were prevented or reversed by copper. The joint administration of cystine and copper alleviated the growth depression caused by excessive molybdenum. The results were discussed in terms of the postulate that the reduced levels of sulfide oxidase in the tissues of rats receiving excessive molybdenum permits an abnormal accumulation of tissue sulfide. This accumulation leads to the formation of tissue copper sulfide and the subsequent appearance of symptoms of copper deficiency.

Siegel et al. (1961) found that the decreased level of the sulfide oxidase activity accompanying molybdate toxicity was attributable largely to the decreased food intake of the animals. Sulfide oxidase activity was considerably higher when diets high in copper were used. This effect appears to be independent of the dietary levels of molybdate, cystine or sulfate. The correlation of enzymatic activity with dietary

supply of copper suggests that the enzyme may be dependent in some way upon copper *in vivo*. Also, the addition of cystine to high-copper diets (with or without molybdate) has been observed to increase sulfide oxidase activity. Since one of the pathways for the metabolism of cystine leads to the formation of sulfide, it is possible that the level of enzyme activity is modified by the availability of its substrate.

Copper Toxicity. Hall *et al.* (1931) fed rabbits a diet containing 2 mgm. of copper acetate per gram. Most of the copper-fed animals showed pigmentation of the liver, mostly in the form of homofusein stored in Kupffer giant cells.

Boughton *et al.* (1934) reported that so-called icterohemoglobinuria that occurred in West Texas was chronic copper poisoning. This condition resulted from the long-continued ingestion of commercial salt mixtures, which contained only relatively small percentages of copper sulfate. Typical cases of the condition were produced experimentally by the feeding of two such commercial mixtures to healthy sheep. Because copper is eliminated very slowly from the body, many animals die from the poisoning for weeks or a few months after the source of copper has been removed. Chronic copper poisoning in sheep is characterized by a yellow discoloration of the tissues and brown to black urine, loss of appetite and weakness.

Marston *et al.* (1948) administered copper thrice weekly to ewes for three and one-half years in doses which provided zero, one, five, 50 and 100 mgm. copper per day. All the individuals receiving no copper became anemic, lost weight and developed marked symptoms of copper deficiency. The supplement of one mgm. copper per day was sufficient to delay the onset of these symptoms, but it failed to increase significantly the

concentration of copper in the blood. Also, this level of copper was insufficient to ensure normal myelin formation in the central nervous system of lambs born of the ewes which had received this treatment for two years prior to conception. The supplement of 5 mgm. copper per day provided sufficient copper for these functions. Dosing with the equivalent of 100 mgm. copper per day resulted in toxicity in which extensive hemolysis was accompanied by icterus. The supplement of 1 mgm. copper per day had no beneficial effect on the character of the wool fleece, and 5 mgm. copper per day was not sufficient in all cases to ensure normal keratinization. The fleeces of the experimental animals which received the equivalent of 50 mgm. copper per day and of 100 mgm. copper per day were normally crimped.

Kidder (1949) in studying copper requirement of cattle and determining the maximum tolerance as well as the minimum requirement, found that some cattle were killed by overdoses of copper sulfate. One 500 pound steer developed chronic copper poisoning and died after receiving a daily drench of 5 gm. copper sulfate for 122 days. Postmortem examination revealed the typical generalized icterus, hemolysis, hemoglobinuria, enlarged kidneys, poorly collapsed lungs, enlarged spleen liver and with a yellowish color with hard surface.

Boyden et al. (1938) fed rat diets which contained zero, 500, 1,000, 2,000, and 4,000 ppm of added copper in the form of $CuSO_4$ and observed slight toxicity symptoms on the 500 ppm diet. Increasing toxicity symptoms were noted on the higher levels. The animals were killed after four weeks and the blood, spleens and livers were analyzed for copper. The copper content of the blood and spleen was increased a maximum of two to five times the normal levels while liver copper increased to a

maximum of 300 times normal.

A reciprocal antagonism between molybdenum and copper, in which the level of dietary molybdenum affects copper metabolism and the level of dietary copper affects molybdenum metabolism, has been demonstrated in sheep, cattle, rats, rabbits and chicks. The current investigations were designed to investigate the possibility of neutralizing the toxicity effects of excessive dietary copper with dietary molybdenum and that of excessive dietary molybdenum by dietary copper.

EXPERIMENTAL PROCEDURE

The compositions of the purified rations (Oltjen et al., 1962a, 1962b, 1962c) are shown in Tables I and II. Ten native lambs averaging 45 pounds, were alloted equally and randomly to five treatments, and fed the basal ration (ration 1), the basal plus 100 ppm copper (ration 2), the basal plus 100 ppm copper plus 10 ppm molybdenum (ration 3), basal plus 1,000 ppm copper (ration 4), and basal plus 1,000 ppm copper plus 100 ppm molybdenum (ration 5). All sheep were individually-housed during this 45-day experiment in wooden stalls, which were designed to eliminate possible trace mineral contamination. The stalls, which were raised 30 inches from the ground, had wooden floors through which were bored many holes three-fourths of an inch in diameter, allowing for disposal of feces and urine. Distilled water, after having passed through a Barnstead Demineralizer containing cation and anion beds, was supplied to the animals in plastic containers. The feed was dispensed once daily in feeders made of wood. Daily feed records and weekly weights were kept on all animals during the entire experiment. Blood samples for hemoglobin determination were removed from the jugular veins of animals on the first

TABLE I

PERCENTAGE COMPOSITION OF THE PURIFIED RATION

Ingredient	Percentage
Starch	29.35
Cerelose	29.35
Solka Floc	30.00
Urea	4.20
Corn Oil	1.00
Mineral Mix	5.00
Polyethylene Resin[a]	1.00
Choline Chloride	0.10
Vitamin Supplement	
Vitamin A Acetate[b]	0.40 gm.
Calciferol (Granular)[c]	0.05 gm.

[a]"Alathon", E. I. Du Pont De NeMours and Co., Inc., Wilmington, Del.

[b]Vitamin A acetate containing 1 million units per gram.

[c]Calciferol (granular) containing 850,000 units per gram.

TABLE II

PERCENTAGE COMPOSITION OF THE MINERAL MIXTURES

Ingredient[a]	Ration 1	Ration 2	Ration 3	Ration 4	Ration 5
K_2CO_3	44.38	44.00	40.40	43.95	39.90
$CaHPO_4$	20.00	20.00	20.00	20.00	20.00
$MgSO_4$	10.00	10.00	10.00	10.00	10.00
NaCL	24.00	24.00	24.00	24.00	24.00
$FeSO_4$	0.85	0.85	0.85	0.85	0.85
$Na_2S_4O_7$	0.25	0.25	0.25	0.25	0.25
$MnSO_4 \cdot H_2O$	0.228	0.228	0.228	0.228	0.228
$ZnSO_4 \cdot 7H_2O$	0.2663	0.2663	0.2663	0.2663	0.2663
$CuCO_3$	0.02	0.40	4.00	0.40	4.00
$Na_2MoO_4 \cdot 2H_2O$	----	----	----	0.05	0.50
CaF_2	0.004	0.004	0.004	0.004	0.004
$CoCL_2 \cdot 6H_2O$	0.0009	0.0009	0.0009	0.0009	0.0009
Na_2SeO_4	0.0005	0.0005	0.0005	0.0005	0.0005
KI	0.0003	0.0003	0.0003	0.0003	0.0003

[a]All minerals were of the analytical reagent grade.

and last days of experiment. An AO Spencer Hb-Meter was used for hemoglobin determinations. Statistical analysis of the data were conducted by the analysis of variance method which was described by Snedecor (1956) and individual comparisons were conducted using the Multiple Range Test (Duncan, 1955).

RESULTS

The results of this experiment are shown in Table III. Differences between sheep consuming rations 1, 2, 3, and 4 were not significant ($P>0.05$) when weight gains and feed efficiencies were considered; however, all sheep gained faster and utilized their feed more efficiently than those consuming ration 5.

DISCUSSION

The sheep receiving ration 5 were in poor condition and exhibited symptoms of molybdenum toxicosis (Underwood, 1956). The addition of 1,000 ppm of copper in the basal diet which contained 0.15 percent sulfur, provided as sulfate, apparently did not overcome the effects of 100 ppm of added molybdenum. In this connection Miller et al. (1956) found that growth inhibition in rats, which was caused by a dietary level of 100 ppm of molybdenum was overcome by 2,200 ppm of sulfate and Halverson et al. (1960) reported that the joint administration of cystine and copper alleviated the depression of growth in rats caused by the excessive level of dietary molybdenum.

It is interesting to note that 1,000 ppm of copper (ration 4) did not affect growth, feed consumption or hemoglobin level of the sheep during this 45-day test. Jones (1954) reported that the experimental

TABLE III

EFFECTS OF VARIOUS DIETARY MOLYBDENUM AND COPPER LEVELS ON GROWTH, FEED EFFICIENCIES AND HEMOGLOBIN LEVELS OF SHEEP (45 DAYS)

Ration Number	Ration	Average Daily Gain (lb.)	Average Feed Required Per Lb. of Gain (lb.)	Average Hb (Gm./100 Ml.)
1	Basal + 5 ppm Cu.	.27 ± .04[a]	7.0 ± 1.0[a]	11.2 ± 0.1[a]
2	Basal + 100 ppm Cu	.21 ± .04	9.4 ± 1.5	12.0 ± 0.5
3	Basal + 100 ppm Cu + 10 ppm Mo	.26 ± .04	7.5 ± 1.1	12.8 ± 0.3
4	Basal + 1,000 ppm Cu	.18 ± .04	10.6 ± 1.8	12.5 ± 0.5
5	Basal + 1,000 ppm Cu + 100 ppm Mo	.02* ± .00	75.0** ± 5.0	13.0 ± 0.1

[a]Standard error

*($P < 0.05$)

**($P < 0.01$)

feeding of approximately 0.6 to 1.0 gm. of copper sulfate daily to sheep produced symptoms of chronic copper poisoning within three to 10 weeks and that toxicity symptoms were brown to black urine, loss of appetite and weight losses. As copper is eliminated from the body at a very slow rate, animals continue to die from chronic copper poisoning for five months after the source of copper has been removed (Boughton and Hardy, 1934). The sheep in the present studies (ration 4) were consuming about one gm. of copper per day and did not exhibit any of these symptoms. These sheep are still under observation (32 days after copper was removed) and no toxicity symptoms have appeared. It is of further interest that Underwood (1956) reported that chronic copper toxicity symptoms were noted in sheep consuming forages containing 50 to 60 ppm of copper. In one area toxicity symptoms were noted when sheep grazed Trifolium subterraneum containing only 10 to 15 ppm of copper; however, the molybdenum level of this forage was only 0.1 to 0.2 ppm. The problem is complex and it would appear that the use of a purified diet, such as the one in the present experiment would be of help in clarifying the problem; however, there could be limitations with this approach: Lewis (1954) found that sulfate is reduced to sulfide in the rumen and Reilley (1961) found that a copper-ammonium complex is readily formed when these ions are mutually present in an aqueous phase. As urea is rapidly hydrolyzed to ammonia and carbon dioxide, the conditions would be optimum for the formation of this complex as well as the formation of poorly soluble cuprous and cupric sulfides; thus, the absorption of dietary copper under these conditions is probably low. In this connection, it should be noted that the sheep on the basal diet, which supplied 5 ppm of copper, apparently had the lowest level of hemoglobin;

there was very little variation in the initial hemoglobin levels, which averaged 13.5 gm./100 ml. of blood. Oltjen and Tillman (1962) also found that when the copper level of the purified diet was increased from 5 ppm to 25 ppm the hemoglobin also was increased 10 percent in a 21-day test.

SUMMARY

Sheep were fed a purified diet in which urea was the source of dietary nitrogen. When copper was added at levels of five, 100 and 1,000 ppm, gains were not affected. When molybdenum was added at 10 ppm, gains of the sheep were not affected; however, when the molybdenum level was increased to 100 ppm, gains of sheep were reduced significantly even though the diet contained 0.15 percent sulfate-sulfur and 1,000 ppm of copper. It was found that the addition of 1,000 ppm of copper to the purified diet was not toxic in this 45-day sheep growth test.

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VITA

Giuma M. Sheriha

Candidate for the Degree of

Doctor of Philosophy

Thesis: MOLYBDENUM IN RUMINANT NUTRITION

Major Field: Animal Nutrition

Biographical:

Born: August 25, 1935 at Kabow, Tripoli, Libya.

Undergraduate Study: Cairo University, 1953-1957.

Graduate Study: Oklahoma State University of Agriculture and Applied Science, 1958-1962.

Date of Final Examination: May, 1962.