



100
641
THS

NITRATE AND NITRITE TOXICOSIS
IN SHEEP

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
ROBERT LEE AMSTER
1970

1118515



~~SEP 18 1972~~ 114

APR 25 1990
9- 114

327

ABSTRACT

Nitrate and Nitrite Toxicosis in Sheep

by Robert Lee Amster

Sheep were fed lettuce which contained relatively high concentrations of nitrate to determine whether methemoglobin formation would occur. Sheep were administered large amounts (3.5 Kg. fresh weight) of lettuce after dehydration of the plant, powdering and then making a paste using distilled water.

The oral administration of lettuce containing 3 grams of nitrate did not produce a noticeable rise in methemoglobin formation. Methemoglobin concentrations caused by oral administration of NaNO_3 and NaNO_2 were used as criteria in evaluating the potential toxicity of nitrate or nitrite in lettuce. The oral administration of 600 mg. $\text{NaNO}_3/\text{Kg.}$ and 40 mg. $\text{NO}_2/\text{Kg.}$ body weight resulted in a 35 to 55 per cent conversion of hemoglobin to methemoglobin. The levels of nitrite (40 mg. $\text{NO}_2/\text{Kg.}$ body weight) required to produce moderate to severe methemoglobinemia in sheep was considerably lower than previously reported.

Since formation of methemoglobin is the major factor in the pathogenesis of nitrate toxicosis and methemoglobin formation was variable in sheep administered NaNO_3 , methemoglobinization of blood in vitro was studied. Results in which NaNO_2 was used in vitro indicated that the variability of methemoglobin formation in sheep is probably due to factors in the rumen rather than in the blood.

NITRATE AND NITRITE TOXICOSIS
IN SHEEP

By
Robert Lee Amster

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1970

Dedicated to

My Family

ACKNOWLEDGEMENTS

I wish to express my gratitude to those who have contributed significantly to the development of this work. Through their efforts and patience the completion of this project has been possible. A most heartwarming thanks to Dr. S. D. Sleight, my major professor, who has made this thesis possible from conception to delivery. I also wish to thank Dr. Langham who has borne the task of guiding me through my hours of histopathology.

I wish to thank Dr. Ries and Paul Rogers of the Horticulture Department for their assistance.

Special acknowledgement is made to my wife, Mary Frances, who assisted in the preparation of this thesis.

Finally I would like to express my gratitude to the United States Air Force for making it possible for me to attend Michigan State University.

TABLE OF CONTENTS

INTRODUCTION	1
REVIEW OF LITERATURE	3
MATERIALS AND METHODS	10
EXPERIMENTAL	14
RESULTS	17
DISCUSSION	33
SUMMARY AND CONCLUSIONS	37
REFERENCES	39
VITA	42

LIST OF FIGURES

Figures	Page
1. Mean methemoglobin values in sheep after oral administration of 150, 275, 400 and 600 mg. NaNO_3 /Kg. body weight.	23
2. Mean methemoglobin values in sheep after oral administration of 20, 30 and 40 mg. NO_2 /Kg. body weight.	27
3. <u>In vitro</u> methemoglobinization of sheep blood induced by NaNO_2 40 mg./100 ml. blood.	29
4. <u>In vitro</u> methemoglobinization of sheep blood induced by NaNO_2 10 mg./100 ml. blood.	30

LIST OF TABLES

Table		Page
1.	Methemoglobin and hemoglobin values of sheep administered powdered lettuce containing nitrite.	18
2.	Methemoglobin and hemoglobin values of sheep given 182.4 Gm. of lettuce containing the equivalent of 3 Gm. of nitrate.	20
3.	Methemoglobin values in sheep after oral administration of 150 mg. NaNO_3 /Kg. body weight.	20
4.	Methemoglobin values in sheep after oral administration of 275 mg. NaNO_3 /Kg. body weight.	21
5.	Methemoglobin values in sheep after oral administration of 400 mg. NaNO_3 /Kg. body weight.	21
6.	Methemoglobin values in sheep after oral administration of 600 mg. NaNO_3 /Kg. body weight.	22
7.	Methemoglobin values in sheep after oral administration of 20 mg. NO_2 /Kg. body weight.	25
8.	Methemoglobin values in sheep after oral administration of 30 mg. NO_2 /Kg. body weight.	25
9.	Methemoglobin values in sheep after oral administration of 40 mg. NO_2 /Kg. body weight.	26
10.	Methemoglobin values in sheep after oral administration of 275 mg. NaNO_3 /Kg. body weight one half hour after oral administration of <u>Ru-Bac</u>	32
11.	Nitrite values of lettuce after <u>E. coli</u> reduction and original nitrate value before reduction.	32

INTRODUCTION

Nitrite induced methemoglobinemia has been observed in man and animals for many years. With the increased use of nitrate and nitrite compounds in food preservatives and fertilizers, an increasing concern has occurred. The public health hazard is compounded by the accumulations of nitrate in plants.

Nitrate in plants is potentially toxic to animals if reduced to nitrite since nitrite causes the conversion of hemoglobin to methemoglobin. Methemoglobin is the ferric porphyrin complex of hemoglobin which is ferrous in its usual state. Methemoglobin is incapable of combining with molecular oxygen and thus cannot participate in the transport of oxygen to the tissues of the body.

Although there are varied causes of circulating methemoglobin, this thesis will deal with methemoglobin caused by nitrate or nitrite toxicosis. It was important to include nitrate because nitrate in plants or food may be a target for bacterial reduction to nitrite. There are several types of bacteria capable of making this reduction including the coliform group which are common contaminants in food and water.

With the increased use of fertilizers and herbicides, it has been found that the nitrate content in some plants may often be extremely high. Excessive nitrate in plants presents a double danger: in the diet of animals it can lead to poisoning, and plants stored in silos can liberate nitrogenous gases that are highly toxic to all animals, including man.

The objective of this research was to determine if the amount of nitrate in commercial lettuce would elicit methemoglobin formation in sheep. A ruminant was chosen since nitrate reduction to nitrite by normal ruminal flora is known to occur. If methemoglobin formation occurred, plants of this nature have a potential toxicity for ruminants and possibly other animals including man.

REVIEW OF LITERATURE

Pathogenesis of Nitrate and Nitrite Toxicosis

Methemoglobin formation is the result of exposure to an agent capable of oxidizing the ferrous porphyrin complex of hemoglobin to the ferric porphyrin complex of methemoglobin. Cohen and Hochstein (1964) found that hydrogen peroxide was generated during the reaction between nitrite and hemoglobin. However, the role of hydrogen peroxide as essential intermediary product in the oxidation process has not been elucidated (Kiese, 1966). Winter (1962) reported that hydroxylamine was formed from nitrite in the blood and was itself capable of converting hemoglobin to methemoglobin.

Bodansky (1951), emphasized that when a methemoglobin-forming compound is administered, methemoglobin accumulates in the blood at a rate characteristic of the compound. Methemoglobin attains a maximum concentration for a variable period of time and then begins to decrease. The shape of the curve is the result of three processes: (a) metabolism of the compound to other substances which do not oxidize hemoglobin, (b) formation of an intermediate compound which reacts directly with hemoglobin to form methemoglobin, and (c) reduction of methemoglobin to hemoglobin by the enzyme systems of the blood. These three processes are continuous and interdependent. Spicer (1950) noted differences in the reaction rate between nitrite and the hemoglobin of various species. Stolk and Smith (1966) found a greater methemoglobin

reductase activity in red blood cells of the mouse and rabbit, whereas the cat, dog and human red blood cells possessed lesser amounts of methemoglobin reductase activity. Methemoglobinemia leads to a hypoxia of all body tissues. Simon et al. (1958) regarded the hypoxic state in tissues and organs as the basis for the pathologic changes associated with nitrate and nitrite toxicosis.

Nitrates in Ruminants

Nitrates are normally converted to nitrite by bacterial or enzymatic action within the digestive tract of the ruminant. Nitrite is the first step in a normal process that converts the nitrate to nitrogen. Methemoglobin formation occurs when the amount of nitrate being ingested produces a greater amount of nitrite than can be utilized. Crawford et al. (1958) found that it was difficult to specify the level of nitrate that may safely be fed to cattle. There is a range of levels from zero dose to maximum safe dose, or LD₀ within which no animals die. Tolerance of nitrate varies from animal to animal as well as from species to species. Therefore, it is impossible to establish the LD₀ with absolute certainty unless every single animal under all possible circumstances is tested. Crawford et al. (1958) also emphasized that the danger to ruminants from nitrate depends on at least three major factors: (a) the concentration of nitrate in the forage, (b) the total quantity of nitrate consumed, and (c) the speed of intake of the toxic forage. Lewis (1951) noted that above a certain concentration of nitrate the rate of reduction of nitrite to ammonia becomes limiting, and consequently nitrite accumulates in the rumen. A bacterial homogenate prepared from rumen liquor collected from a cow fed 30 Gm. NaNO₃/45 Kg. body weight

reduced both nitrate and nitrite faster than a similar homogenate prepared from a control cow (Davison et al., 1962). Crawford et al. (1960) stated that animals with stomach contents that are acidic (pig, dog, rat, man) do not normally harbor the intestinal bacteria that convert nitrate to nitrite. As a result, they are more unlikely to be poisoned by nitrate. However, in an abnormal situation, such as bacterial gastroenteritis, bacteria may be present that would convert the nitrate to nitrite.

Methemoglobinemia in Man Caused by Nitrates or Nitrites

Comly (1945) reported that many rural drinking water reservoirs for human consumption were contaminated with coliform organisms. He described cyanosis in infants caused by nitrates in well water which contained the coliform organisms. Water samples from 20 per cent of 91 dug wells in Iowa had nitrate levels above 65 parts per million (ppm). Comly suggested that well water used in infant feeding should have a nitrate content no higher than 10 or, at the most, 20 ppm. According to the United States Public Health Service (1962) water containing 45 ppm nitrate-nitrogen ($\text{NO}_3\text{-N}$) has been considered hazardous for use in feeding infants. Hölscher and Natzschka (1964) reported cases of methemoglobin formation in infants fed spinach purée containing high levels of nitrite.

The age of the child is one of the most important considerations in nitrate toxicity (Phillips, 1967). Keohane and Metcalf (1960) reported that hemoglobin from fetuses and the newborn is more sensitive to the action of methemoglobin forming agents such as nitrites. Sinha (1968) found blood of fetal guinea pigs more susceptible to methemoglobin formation than blood from adults.

Ross (1963) reported that premature infants are more susceptible to methemoglobin formation than infants born at term.

Metcalf (1961) reported that hemoglobin sensitivity to methemoglobin forming agents extends to pregnant women, patients with carcinomata and to patients with some other conditions. Using in vitro studies, Metcalf, found that the nitrite conversion rates were significantly faster in children, pregnant women and adults with definite carcinomata. Keohane and Metcalf (1960) obtained little evidence as to the cause of the differing sensitivity of cells to nitrite in children and adults. There were four possibilities postulated. The difference may be in the plasma, in the cell membrane, in the hemoglobin itself or in some other intercellular constituent. Burden (1960) demonstrated that the higher gastric pH and greater relative fluid intake in infants facilitates the bacterial reduction of nitrate to nitrite.

Sodium nitrate and sodium nitrite are incorporated in some fish and meat products at levels of 200 and 500 ppm, respectively, as preservatives (Fassett, 1966). Orgeron et al. (1957) described several outbreaks of nitrite poisoning following the accidental incorporation of excessive amounts of nitrate and nitrite mixtures in meat products.

Methemoglobinemia in Animals

In a later report by Metcalf (1962) in work with rats, the reaction time to form methemoglobin of young rats as compared to that for older animals seemed to be more closely related to growth rate than to puberty. From this he concluded that a part of the diet may be involved. Of all the individual components of the diet tested, riboflavin alone, was capable of markedly slowing the reaction time of the young and pregnant rats. Riboflavin occurs in the body as a constituent of many enzymes

and enzyme systems concerned with electron transfer. Metcalf suggested that a flavoprotein enzyme could delay the oxidation of the hemoglobin ferrous iron by nitrites, by acting as an electron acceptor until exhausted. Riboflavin requirements are especially great during rapid growth, pregnancy and debilitated conditions.

Feeding trials by Crawford et al. (1960) with dairy cattle on a typical ration, indicated that a rise in methemoglobin was not significant at levels of nitrate in forage up to 15 Gm. per 45 Kg. of body weight. A sharp increase in methemoglobin was not attained until levels greater than 25 Gm. of nitrate per 45 Kg. of body weight were given. Death may not occur until amounts greater than 25 Gm. of nitrate per 45 Kg. of animal weight are consumed. It is impossible, however, to predict the exception to the rule. In their work with pregnant dairy cattle, Simon et al. (1959) concluded that the concentrations of methemoglobin varied among animals within groups and in individual animals on different days.

Setchell and Williams (1962) reported that sudden death in pregnant ewes occurred due to chronic ingestion of food containing 2 per cent potassium nitrate. However, the sheep had only a slight rise in methemoglobin and no determination was made as to the cause of death. Protection against the methemoglobinemia induced by orally administered sodium nitrate was obtained by drenching sheep twice daily with a sucrose solution or by the addition of corn to a forage ration (Emerick et al., 1965).

Nitrate in Plants

The increased uptake of nitrate in plants is affected by limited moisture supply, low light intensity and low temperatures. This is

probably because the growth of the plant is decreased to a greater degree than is the nitrogen uptake (Breniman et al., 1961). Concentrations of nitrate high enough to be dangerous indicate that the conversion process has lagged behind the absorption process. The presence of nitrate in large quantities due to high nitrogen fertilization is a ready source for plant uptake. Apparently the accumulation of nitrate is not harmful to the plant (Crawford et al., 1960). Marked differences are found in nitrate accumulation between plant species as well as between different parts of the plant. The nitrate content is highest in the stems, intermediate in the leaves and lowest in seeds. Immature plants generally contain more nitrates than mature plants (Crawford et al., 1960). Davison (1966) reported that the species of plant is an important factor in suspected nitrate poisoning. Corn, oats and other small grains, sudan grass and other sorghums, and many common weeds may accumulate large quantities of nitrate.

The utilization of nitrate by the plant is postulated to occur by the pathway of nitrate $\xrightarrow{\text{nitrate reductase}}$ nitrite $\xrightarrow{\text{nitrite reductase}}$ ammonia. Nitrate reductase appears to be the regulator of nitrates in this system (Hageman et al., 1962). When fertilizers that supply nitrate are added to soils, the nitrate is rapidly absorbed by the growing crop (Crawford et al., 1960). Ordinarily this nitrate is reduced quickly for incorporation into amino acids which are vital to the life processes of the plant. The field application of simazine [2-chloro-4, 6-bis(ethylamino) -s- triazine], a herbicide, increased the total nitrogen level of tolerant plant species (Ries et al., 1963). In a later study by Ries et al. (1968) 0.23 Kg.

of simazine per acre caused more than an eightfold increase in nitrate content of dry rye grass forage. The protein content was increased in rye grass forage, rice foliage, alfalfa forage, peas, beans and oats. Simazine application to an established alfalfa stand increased the protein content but did not appreciably increase the nitrate level.

11

MATERIALS AND METHODS

General Plan and Considerations

The primary objective of this research was to determine if nitrate in lettuce was capable of causing methemoglobin formation in vivo. The amount of nitrate present in lettuce was determined so that a dosage equivalent to this could be given as NaNO_3 . In another experiment an attempt was made to reduce the nitrate in lettuce to nitrite by means of Escherichia coli. In this manner it would be possible to determine if the reduction of nitrate to nitrite in the lettuce would cause more methemoglobin formation than that caused by unreduced nitrate in lettuce. It was necessary to determine the amount of methemoglobin formed after the administration of NaNO_3 and NaNO_2 . These results were compared with those obtained after the administration of lettuce and this information made it possible to determine the percentage of nitrate in lettuce, or chemical, that would require reduction to nitrite to cause a methemoglobinemia. Thus, 3 Gm. of nitrate present in lettuce could induce the same amount of methemoglobin formation as 3 Gm. of NaNO_3 . It was desired to determine if a known amount of nitrate in lettuce reduced to nitrite could produce a methemoglobinemia comparable to an equivalent amount of NaNO_2 . Preliminary tests with the sheep used in this study indicated that there was a variability among the sheep as to the amount of methemoglobin formed after being administered NaNO_3 . Experiments using NaNO_2 in vitro were conducted to determine if the variability was due to individual blood characteristics or ruminal differences in the reduction of nitrate.

The fact that the rumen bacteria are able to reduce nitrate to nitrite suggested a study on the effect of increasing the population of

ruminal bacteria. An experiment was then designed to increase the number of bacteria present in the rumen in order to enhance the reduction of nitrate to nitrite by the normal ruminal flora. A known amount of NaNO_3 was used as the nitrate source in the altered ruminal flora experiment.

Plasma nitrate and plasma nitrite values were determined during the studies so that their appearance and disappearance in the blood could be followed. Hemoglobin values were determined mainly as a means of obtaining methemoglobin values as per cent hemoglobin.

Techniques, Chemicals and Care and Management of the Sheep

Nine mature, crossbred sheep were used in the study. Fecal samples were checked for internal parasites and the sheep were treated with thiabendazole* at the beginning of the experiment. They were kept in a field lot with shelter from the rain and cold and hay was fed during the winter months. Nitrate levels were found to be insignificant in the hay, pasture and water.

Ten milliliters of blood obtained from the external jugular vein were required for plasma nitrate, plasma nitrite, methemoglobin, and hemoglobin determinations. Heparinized** 10 ml. disposable syringes with 2.5¹/₄ cm. disposable 20-gauge needles were used.

Methemoglobin values were determined photometrically*** using the Evelyn and Malloy (1938) procedure. As soon as possible the blood

*Equizole - Merck Chemical Division, Rahway, N. Y.

**Heparin (ammonium salt) - Biological Research Inc., St. Louis, Mo.

***Coleman Model 6D Junior Spectrophotometer, Coleman Instruments Inc.,
42 Madison Street, Maywood, Ill.

(0.1 ml.) was mixed in M/60 phosphate buffer* in order to prevent reduction of methemoglobin (Sleight and Sinha, 1968). Hemoglobin values were determined by the cyanmethemoglobin method (Benjamin, 1961). Optical densities were recorded by the use of a spectrophotometer.

To prepare protein-free plasma for nitrite and nitrate determinations the blood was centrifuged soon after collection and the plasma was removed. The procedure of Diven et al. (1962) was followed. The nitrate determination had the following modification: instead of the addition of powdered zinc, a small amount of root nodule bacterioid was added. The determination of plasma nitrate and nitrite value was performed by using the color development procedure as described by Diven et al. (1962).

For all experiments a fresh solution of sodium nitrate (NaNO_3)** and sodium nitrite (NaNO_2 *** was prepared. The dosages of NaNO_2 were determined as the amount of nitrite given.

The core was removed from 40 heads of lettuce, with a fresh leaf weight of 38 Kg.. The lettuce leaves were spread in a 60 C. drying room for 40 hours. The dry weight of the leaves was 2.05 Kg., which was 5.4 per cent of the fresh weight. The dry leaves were crushed and then ground in a Wiley Mill**** using a 1 mm. mesh screen, the final form being

* A M/15 stock phosphate buffer (pH 6.6) was prepared by dissolving 9.0 Gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 5.7 Gm. of anhydrous KH_2PO_4 in water and diluting to 1 liter. Three volumes of water were added to 1 volume of the stock buffer to prepare M/60 buffer.

**Sodium Nitrate Crystal (NaNO_3) - "Baker Analyzed" Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.

***Sodium Nitrite Crystal (NaNO_2) - "Baker Analyzed" Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.

****Wiley Mill - Mill, Laboratory, Wiley, Intermediate Model-A.

a coarse powder of lettuce. The lettuce was obtained from Desert Products Inc., Holtville, California.

Determinations of nitrate and nitrite in lettuce were made by using the Lowe and Hamilton procedure (1967). The nitrite determinations had the following modification: distilled water was added to the sample instead of bacterioids as nitrite was already present.

Escherichia coli culture was used as a reducing bacteria to obtain nitrite from nitrate in lettuce. A culture was obtained by inoculating a 1,000 ml. volume of trypticase soy broth* with a nonpathogenic E. coli, then incubating in a 37 C. culture room for 36 hours. The growth was exuberant as evidenced by the milky color of the broth when stirred. Three (186 Gm.) samples of powdered lettuce were placed into three 2,000 ml. Erlenmeyer flasks. Distilled water was added to the powdered lettuce and E. coli mixture to form a slurry. The flasks were capped with gauze cotton and incubated at 37 C. After 18 hours of incubation 0.5 ml. of penicillin/streptomycin** was added to kill the E. coli. The reduced lettuce was refrigerated immediately and used within two hours. A sample of slurry containing approximately 2 Gm. lettuce (dry weight) was taken for nitrate and nitrite analysis.

* trypticase soy broth - BBL, Division of BioQuest, Division of Becton Dickinson and Company, Cockeysville, Maryland 21030

P.D.C. - procaine penicillin G in dihydrostreptomycin solution, Jensen-Salsberry Laboratories, Division of Richardson-Merrell Inc., Kansas City, Mo.

1

EXPERIMENTAL

Determinations of Levels of Methemoglobin, Hemoglobin, Plasma Nitrate and Nitrite After Administration of Lettuce Containing Known Quantities of Nitrate

Two sheep, weighing approximately 40 Kg. each, were randomly selected and each given 182.4 Gm. of lettuce by stomach tube. The 182.4 Gm. of lettuce represented 3 Gm. of nitrate. The powdered lettuce was suspended in 3 liters of distilled water and then pumped into the rumen. After the lettuce had been administered, blood samples were obtained at 30 minutes, 1, 2 and 5 hours for methemoglobin, hemoglobin, plasma nitrate and nitrite determinations. A third sheep was given the same amount of lettuce by way of a large dose syringe. The same blood studies were performed. A blood sample was obtained prior to administration of the lettuce to the sheep.

Determinations of Levels of Methemoglobin, Hemoglobin and Plasma Nitrite After Administration of Lettuce Containing Known Quantities of Nitrite

Three sheep weighing approximately 40 Kg. each, were randomly selected and given 182 Gm. of lettuce in which it had been attempted to reduce the nitrate to nitrite using an E. coli culture. The lettuce was administered to the sheep by way of a large dose syringe. A fourth animal was not given any lettuce but was used as a control animal. Blood samples were obtained before lettuce administration and at 30 minutes, 1, 1.5, 2, 3, 4 and 5 hours. The nitrite values are given in the footnote of Table 1.

Determinations of Levels of Methemoglobin, Hemoglobin, Plasma Nitrate and Nitrite After Oral Administration of NaNO_3

Four experiments, 1, 2, 3 and 4 were conducted using four sheep each, randomly selected and weighed before each NaNO_3 administration. The NaNO_3 was dissolved in distilled water to make a 10 per cent solution. The NaNO_3 dosages were given at the rate of 150, 275, 400 and 600 mg./Kg. body weight in experiments 1 through 4 respectively. The NaNO_3 solution was given orally by using 50 ml. disposable syringes. The use of these syringes allowed drenching without loss of any solution. Blood samples were obtained before NaNO_3 administration and at 1, 2, 3, 4, 5 and 6 hours for all studies and 8.5 hours for the sheep given 400 mg./Kg. body weight, and 8, 10 and 12 hours for the sheep given 600 mg. NaNO_3 /Kg. body weight.

Determinations of Levels of Methemoglobin, and Plasma Nitrite After Oral Administration of NaNO_2

Experiments 1, 2 and 3 were conducted using four sheep each, randomly selected and weighed before each NaNO_2 administration. The NaNO_2 dosages were given at the rate of 20, 30 and 40 mg. NaNO_2 /Kg. body weight in experiments 1 through 3 respectively. The NaNO_2 solution was given orally by using 10 ml. disposable syringes. Blood samples were obtained before NaNO_2 administration and at 30 minutes, 1, 2, 3, 4 and 5 hours for the group given 20 mg. NO_2 /Kg.; 30 minutes, 1, 2, 3, 4, 5, 6 and 7 hours for the group given 30 mg. NO_2 /Kg.; and 30 minutes, 1, 2, 3, 4, 6 and 8 hours for the group given 40 mg. NO_2 /Kg. body weight.

Methemoglobinization of Sheep Blood Induced by NaNO_2 (in vitro)

Five milliliters of heparinized blood was added to test tubes (15 by 125 mm.) then placed in a 37 C. water bath. A 1 per cent solution of the NaNO_3 was added to the blood so that the tubes contained the equivalent of 10 mg. and 40 mg. per 100 ml. of blood. During sampling, the tubes were slowly agitated in the water bath in order to keep the red blood cells in suspension. During sampling, the tubes were slowly inverted several times to also facilitate red blood cell suspension. Hemoglobin and methemoglobin were determined at 1, 10, 25, 45, 60 and 90 minutes when 40 mg. of NaNO_2 /100 ml. of blood was used and 0, 20, 40, 60 minutes, 1.5, 2, 2.5, 3, 4, 5, 6, 7 and 10 hours when 10 mg. NaNO_2 /100 ml. of blood was used.

The samples of blood to which 10 mg. of NaNO_2 was added were allowed to remain in the water bath for a total period of 10 hours for reduction to hemoglobin.

Determinations of Levels of Methemoglobin, Plasma Nitrate and Nitrite After Oral Administration of NaNO_3 Following Ru-Bac Administration

To introduce additional bacteria to the ruminal contents four sheep were given Ru-Bac* one half hour before the oral administration of 275 mg. NaNO_3 /Kg. body weight. The dosage of Ru-Bac for each sheep was $1\frac{1}{2}$ teaspoons in 10 ml. of distilled water given as a drench. Blood samples were obtained before NaNO_3 administration and at 1, 2, 3, 4, 5, 6 and 7 hours after administering the NaNO_3 .

* Ru-Bac - Ru-Bac with enzymes powder, Haver-Lockhart Laboratories, Kansas City, Mo.

RESULTS

In this thesis all methemoglobin values will be expressed as the percentage of hemoglobin oxidized to methemoglobin.

Results of Nitrate Determinations in Lettuce

The average value of nitrate in lettuce was 16,426 micrograms per Gm. (dry weight) of lettuce. With this figure it was calculated that 182.4 Gm. of lettuce (dry weight) would be equivalent to 3 Gm. of nitrate.

Determinations of Levels of Methemoglobin, Hemoglobin, Plasma Nitrate and Nitrite After Administration of Lettuce Containing Known Quantities of Nitrite and Nitrate

The three sheep that were administered lettuce containing approximately 50 mg. of nitrite and the control sheep not given lettuce did not have an appreciable rise in methemoglobin (Table 1). There was a smaller amount of nitrite present in the lettuce than had been expected.

The three sheep that were administered lettuce containing the equivalent of 3 Gm. of nitrate did not have an increase in methemoglobin (Table 2). The nitrate in the lettuce was not toxic even in the large quantity represented by 182 Gm. dry lettuce (3,370 Gm. fresh weight).

All hemoglobin values remained within the normal range.

Determinations of Levels of Methemoglobin, Hemoglobin, Plasma Nitrate and Plasma Nitrite After Oral Administration of NaNO_3

In the first experiment the dosage was 150 mg. of NaNO_3 /Kg. body weight. Blood samples collected at zero time, 30 minutes and hourly

Table 1. Methemoglobin and hemoglobin values of sheep administered powdered lettuce containing nitrite.

Time	Sheep 19 44 Kg. *		Sheep 32 37 Kg. *		Sheep 49 41 Kg. *		Sheep 172 45 Kg. *	
	Hgb. Gm./ 100 ml.	% MetHb.	Hgb. Gm./ 100 ml.	% MetHb.	Hgb. Gm./ 100 ml.	% MetHb.	Hgb. Gm./ 100 ml.	% MetHb.
0	12.2	3.1	11.3	3.3	13.0	3.2	12.1	1.1
30 min.	11.3	3.3	11.2	3.1	12.3	3.8	12.8	1.0
1 hr.	10.9	3.8	11.0	4.7	11.9	4.1	12.6	1.2
1.5 hr.	10.9	3.8	10.8	5.2	11.3	4.6	12.4	0.7
2 hr.	10.6	3.2	10.6	4.4	11.9	3.9	12.4	1.9
3 hr.	10.3	1.8	10.9	3.4	11.9	2.0	12.3	0.0
4 hr.	10.9	0.8	11.2	0.4	12.0	1.1	12.3	0.0
5 hr.	10.9	0.8	10.9	0.0	12.0	2.0	12.4	0.4

* Sheep 19 1.10 mg. NO₂/Kg. body wt.
 Sheep 32 1.66 mg. NO₂/Kg. body wt.
 Sheep 49 1.07 mg. NO₂/Kg. body wt.
 Sheep 172 Control, was not administered lettuce

through the sixth hour did not contain any distinct change in methemoglobin (Table 3). The highest value of nearly 5 per cent methemoglobin is not appreciably greater than the normal range of 0 to 3.5 per cent for this group of sheep.

The second study, in which the dosage was 275 mg. $\text{NaNO}_3/\text{Kg. body weight}$, revealed a low increase in circulating methemoglobin in one animal (Table 4). The methemoglobin value for the one sheep reached a maximum of 12.3 per cent, the remaining three sheep were within the normal range of 1 to 3.5 per cent.

In the third experiment the dosage was increased to 400 mg. $\text{NaNO}_3/\text{Kg. body weight}$. A moderate rise of 5 to 24 per cent methemoglobin was attained (Table 5).

The fourth study in which the dosage was increased to 600 mg. $\text{NaNO}_3/\text{Kg. body weight}$ there was an appreciable increase in methemoglobin to 19 to 46 per cent (Table 6).

In these 4 experiments there was an indication that the greater the methemoglobin values the greater the length of time required to attain maximum circulating methemoglobin (Figure 1). In the comparison of the means for these 4 studies, 19 to 31 per cent methemoglobin values were attained 6 to 8 hours after the administration of 600 mg. $\text{NaNO}_3/\text{Kg. body weight}$. A peak of 10 per cent methemoglobin was attained 4 hours after the administration of 400 mg. $\text{NaNO}_3/\text{Kg. body weight}$.

Determinations of Levels of Methemoglobin, and Plasma Nitrite After Oral Administration of NaNO_2

In the first study the dosage was 20 mg. $\text{NO}_2/\text{Kg. body weight}$. The highest methemoglobin concentration (7.25 to 8.25 per cent) was present at one hour and the value then steadily decreased (Table 7).

Table 2. Methemoglobin and hemoglobin values of sheep given 182.4 Gm. of lettuce containing the equivalent of 3 Gm. of nitrate.*

Time	Sheep 35 39 Kg.		Sheep 173 43 Kg.		Sheep 19 40 Kg.	
	Hgb. Gm./ 100 ml.	% MetHb.	Hgb. Gm./ 100 ml.	% MetHb.	Hgb. Gm./ 100 ml.	% MetHb.
0	12.6	.21	12.2	.13	10.5	.00
30 min.	12.8	.21	11.0	.21	11.2	.00
1 hr.	11.4	.00	11.5	.00	10.5	.00
2 hr.	12.6	.08	11.0	.13	11.4	.00
5 hr.	11.2	.00	11.0	.00	10.5	.00

*Approximately 75 mg. NO_3 /Kg. body weight.

Table 3. Methemoglobin values in sheep after oral administration of 150 mg. NaNO_3 /Kg. body weight.

Animal No. Wt.		Methemoglobin (%) at Intervals							
		0 min.	30 min.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
19	37 Kg.	2.4	3.7	2.0	2.7	2.5	0.0	0.0	3.6
165	49 Kg.	3.1	2.4	0.0	2.0	1.1	1.0	1.1	2.5
172	37 Kg.	0.0	2.9	0.0	3.2	1.0	3.1	3.1	1.0
173	48 Kg.	0.0	0.0	0.0	4.8	3.9	3.0	2.6	2.6
Mean		1.4	2.2	0.5	3.2	2.1	1.8	1.7	2.4
S. D.		1.6	1.6	1.0	1.0	1.4	1.5	1.4	1.0

Table 4. Methemoglobin values in sheep after oral administration of 275 mg. NaNO_3 /Kg. body weight.

Animal No.	Wt.	Methemoglobin (%) at Intervals						
		0 min.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
19	34 Kg.	1.3	2.0	1.9	1.0	2.0	0.0	1.1
32	30 Kg.	1.0	1.0	1.4	3.0	3.0	4.0	1.1
165	50 Kg.	0.0	1.5	1.1	0.0	0.0	0.0	0.0
172	37 Kg.	2.3	6.6	12.3	11.2	10.6	2.8	0.5
Mean		1.2	2.8	4.2	3.8	3.9	1.7	0.7
S. D.		0.9	2.6	5.4	5.1	4.6	2.0	0.5

Table 5. Methemoglobin values in sheep after oral administration of 400 mg. NaNO_3 body weight.

Animal No.	Wt.	Methemoglobin (%) at Intervals							
		0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	8.5 hr.
167	45 Kg.	0.5	4.2	14.1	14.1	7.9	6.5	0.0	4.0
175	45 Kg.	1.1	5.4	6.1	17.3	22.9	23.8	22.7	8.0
35	34 Kg.	3.7	1.3	5.6	1.4	1.4	2.8	1.4	4.2
49	36 Kg.	0.9	4.9	3.2	2.3	2.8	2.3	1.4	6.4
Mean		1.6	4.0	7.3	8.8	10.3	8.9	6.4	5.7
S. D.		1.5	1.9	4.7	8.1	10.0	10.2	10.9	1.9

Table 6. Methemoglobin values in sheep after oral administration of 600 mg. NaNO_3 /Kg. body weight.

Hour	Methemoglobin (as % hemoglobin)				Mean	Standard Deviation
	Animal No. 35 39 Kg.	Animal No. 49 35 Kg.	Animal No. 167 50 Kg.	Animal No. 175 45 Kg.		
0	2.9	0.5	0.0	0.0	0.9	1.5
1	3.2	3.7	3.6	5.7	4.1	1.3
2	5.4	7.2	15.5	12.4	10.1	4.6
3	7.0	10.7	22.7	12.7	13.3	6.7
4	10.5	9.4	27.4	16.8	16.0	8.3
5	10.7	9.3	37.3	20.4	19.4	12.9
6	19.6	14.9	46.4	23.2	26.0	14.0
8	34.7	18.8	40.5	29.4	30.9	9.2
10	34.7	14.5	22.7	30.6	25.6	8.9
12	17.3	8.2	7.9	15.0	12.1	4.8
48	0.5	2.8	1.0	1.4	1.4	1.0

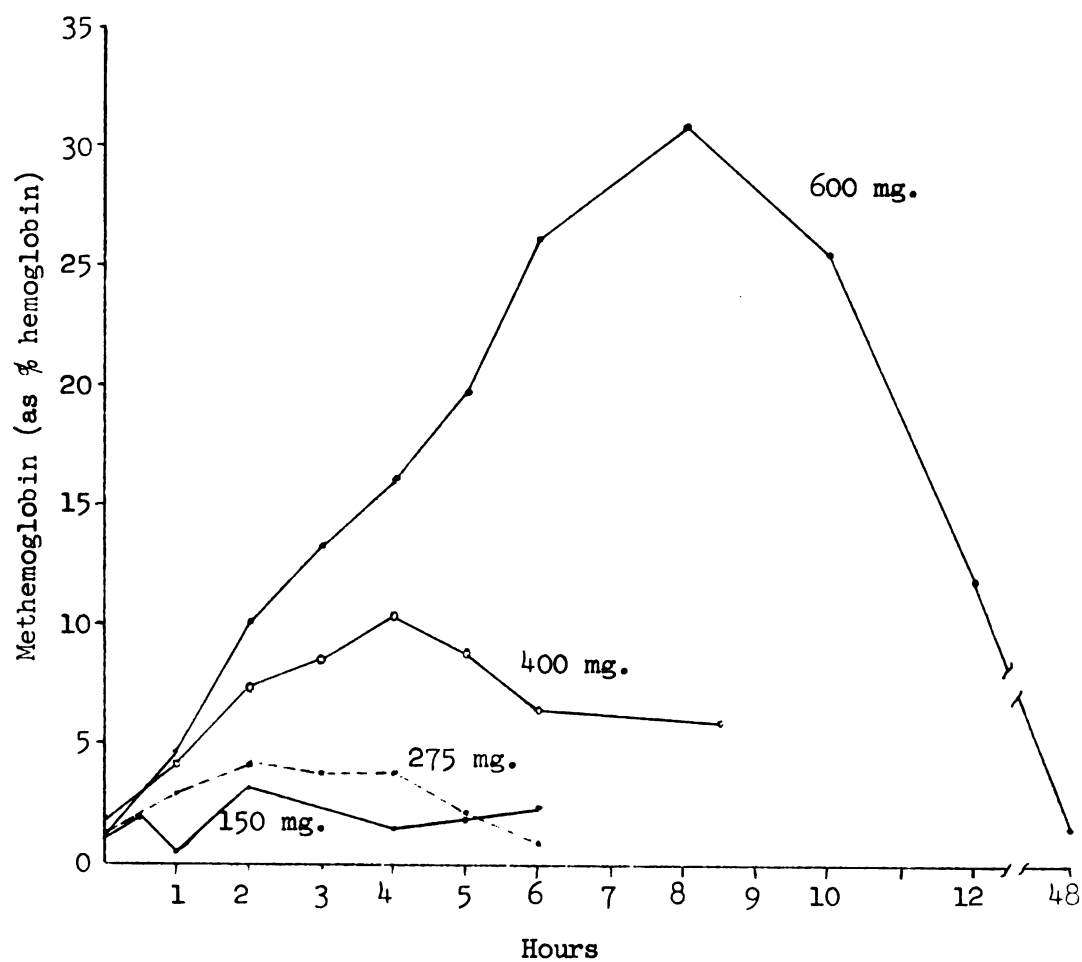


Figure 1. Mean methemoglobin values in sheep after oral administration of 150, 275, 400 and 600 mg. NaNO_3 / Kg. body weight.

In the second study the dosage was 30 mg. NO_2 /Kg. body weight. Three sheep had maximum methemoglobin values of 14 to 24 per cent while a fourth sheep had a considerably higher methemoglobin value of 40 per cent (Table 8). In this study the maximum methemoglobin values were attained at approximately two hours and had decreased by the fourth hour.

In the third study the dosage was 40 mg. NO_2 /Kg. body weight. Individual variation manifested itself in that three sheep had maximum methemoglobin values of 64 to 82 per cent and the fourth sheep had a considerably lower methemoglobin value of 17 per cent (Table 9). In this study the maximum methemoglobin values of 17 to 82 per cent were attained at approximately two hours and had decreased significantly by the eighth hour. During the study the sheep that had the 82 per cent methemoglobin value became weak one hour after NaNO_2 administration. Two hours after NaNO_2 administration this sheep was down and became semicomatose for one and one half hour. After being down for two and one half hours this sheep got up and walked about, but wobbly. Six hours after NaNO_2 administration this sheep appeared normal. During the semicomatose period the sclera had the characteristic chocolate color as seen in nitrite toxicity.

The comparison of the mean values for these 3 experiments indicates that the 30 to 40 mg. nitrite dosages produced a maximum circulating methemoglobin value one hour later than that of the 20 mg. nitrite dose (Figure 2). In these 3 studies the hemoglobin values remained within the normal range.

Table 7. Methemoglobin values in sheep after oral administration of 20 mg. NO_2 /Kg. body weight.

Animal No.	Wt.	Methemoglobin (%) at Intervals						
		0 min.	30 min.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
19	35 Kg.	4.0	6.5	7.7	4.4	2.1	2.3	2.3
172	38 Kg.	1.9	7.5	7.5	3.6	1.8	1.8	0.7
167	45 Kg.	2.0	4.5	7.2	2.3	0.4	1.3	0.0
32	30 Kg.	0.8	2.8	8.3	4.0	3.5	0.4	0.8
Mean		2.2	5.3	7.7	3.6	2.0	1.5	1.0
S. D.		1.4	2.1	0.5	0.9	1.7	0.9	1.0

Table 8. Methemoglobin values in sheep after oral administration of 30 mg. NO_2 /Kg. body weight.

Hour	Methemoglobin (as % hemoglobin)				Mean	Standard Deviation
	Animal No. 19 34 Kg.	Animal No. 32 29 Kg.	Animal No. 35 34 Kg.	Animal No. 167 45 Kg.		
0	4.3	6.0	2.8	7.3	5.1	1.9
0.5	16.9	8.2	20.6	9.8	13.6	5.9
1	23.7	13.7	33.6	15.0	21.5	9.0
2	20.0	12.7	40.2	20.7	23.4	11.8
3	11.9	4.8	40.4	8.0	16.3	8.9
4	7.6	5.0	20.2	4.3	9.3	7.4
5	3.1	3.1	13.5	2.2	5.5	5.4
6	0.5	N.D.	10.3	N.D.	5.3	4.0
7	0.9	N.D.	4.9	N.D.	2.9	1.7

Table 9. Methemoglobin values in sheep after oral administration of 40 mg. NO₂/Kg. body weight.

Animal No.	Wt.	Methemoglobin (%) at Intervals							
		0 hr.	0.5 hr.	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.	8 hr.
49	36 Kg.	3.5	44.2	64.8	81.6	77.9	71.0	55.8	31.5
165	48 Kg.	5.0	40.7	55.9	64.4	57.0	54.4	28.9	6.4
173	49 Kg.	3.8	13.3	16.9	11.9	7.1	4.3	2.3	5.4
175	45 Kg.	2.7	40.3	56.0	68.8	62.1	60.5	32.8	7.6
Mean		3.8	34.8	48.4	56.7	51.3	50.5	30.0	12.7
S. D.		1.0	11.8	21.4	30.7	30.6	30.3	21.9	12.5

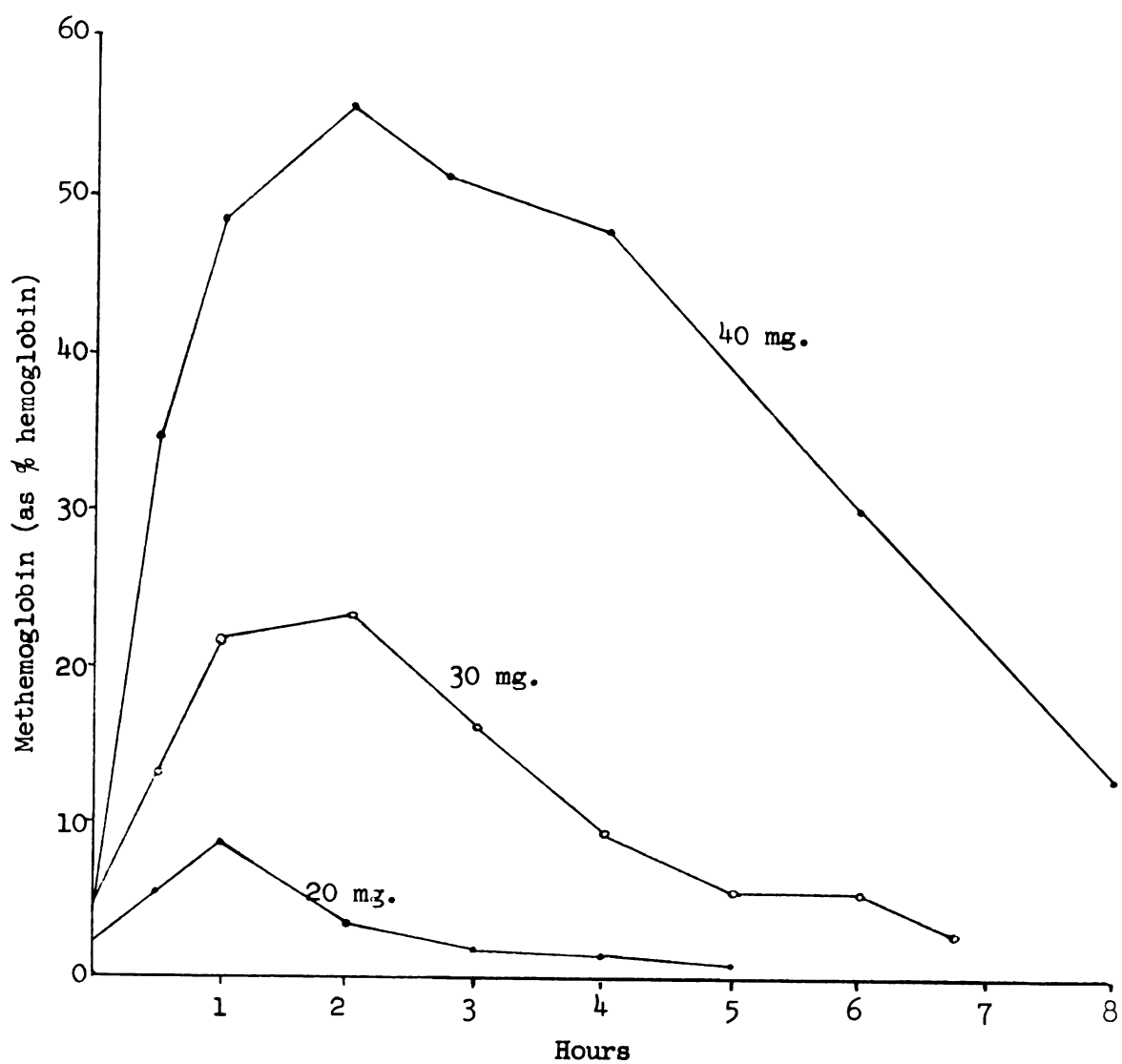


Figure 2. Mean methemoglobin values in sheep after oral administration of 20, 30 and 40 mg. NO_2 /Kg. body weight.

Methemoglobinization of Sheep Blood by NaNO_2 (in vitro)

The methemoglobin level in blood containing NaNO_2 (40 mg./100 ml.) reached a peak of 86 to 94 per cent after a period of 90 minutes (Figure 3). The methemoglobin level in blood containing NaNO_2 (10 mg./100 ml.) reached a peak of 25 to 29 per cent after a period of two and a half hours and returned to less than 5 per cent by the end of 10 hours (Figure 4). The hemoglobin values were relatively well duplicated. As shown in Figures 1 and 2 the methemoglobin values were relatively well grouped. It is concluded that the in vivo methemoglobin variabilities are probably due to ruminal differences.

Determination of Levels of Methemoglobin, Plasma Nitrate and Nitrite After Oral Administration of NaNO_3 Following Ru-Bac Administration

In this study Ru-Bac was given one half hour before NaNO_3 dosing. Three to five hours after NaNO_3 administration three sheep had methemoglobin values moderately higher than normal, the maximum values being 7 to 16 per cent (Table 10). The hemoglobin values remained within the normal range.

In comparing the tables showing the methemoglobin values for sheep given 275 mg. NaNO_3 /Kg. body weight without Ru-Bac (Table 4) and those given 275 mg. NaNO_3 /Kg. body weight with Ru-Bac (Table 10) there is an indication that the apparent enhancement by Ru-Bac of the reduction of nitrate to nitrite is due mainly to results for 1 sheep (#19).

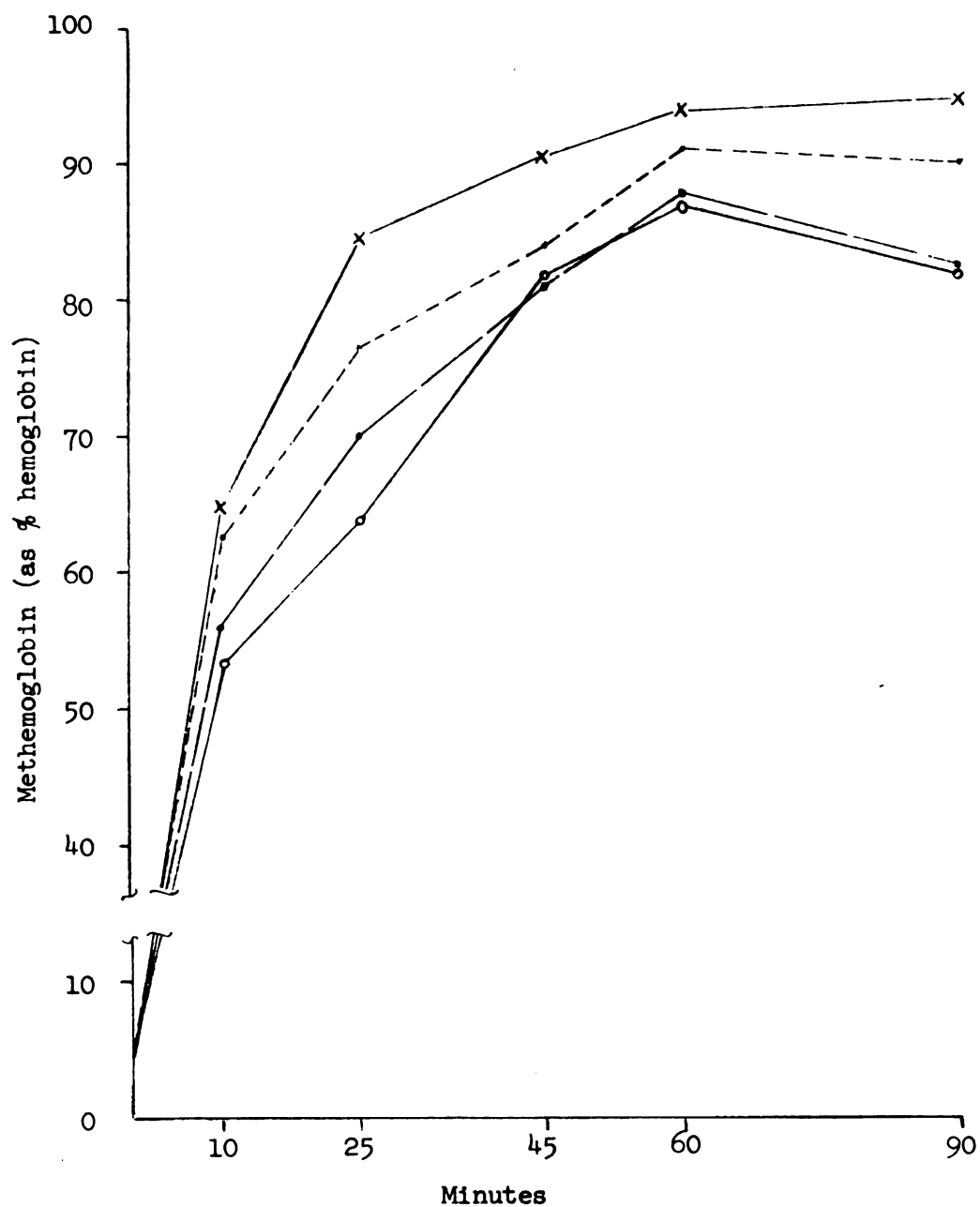


Figure 3. In vitro methemoglobinization of sheep blood induced by NaNO_2 40 mg./100 ml. blood. x ——— x sheep 173, - - - - - sheep 172, ———— sheep 165, o ——— o sheep 167.

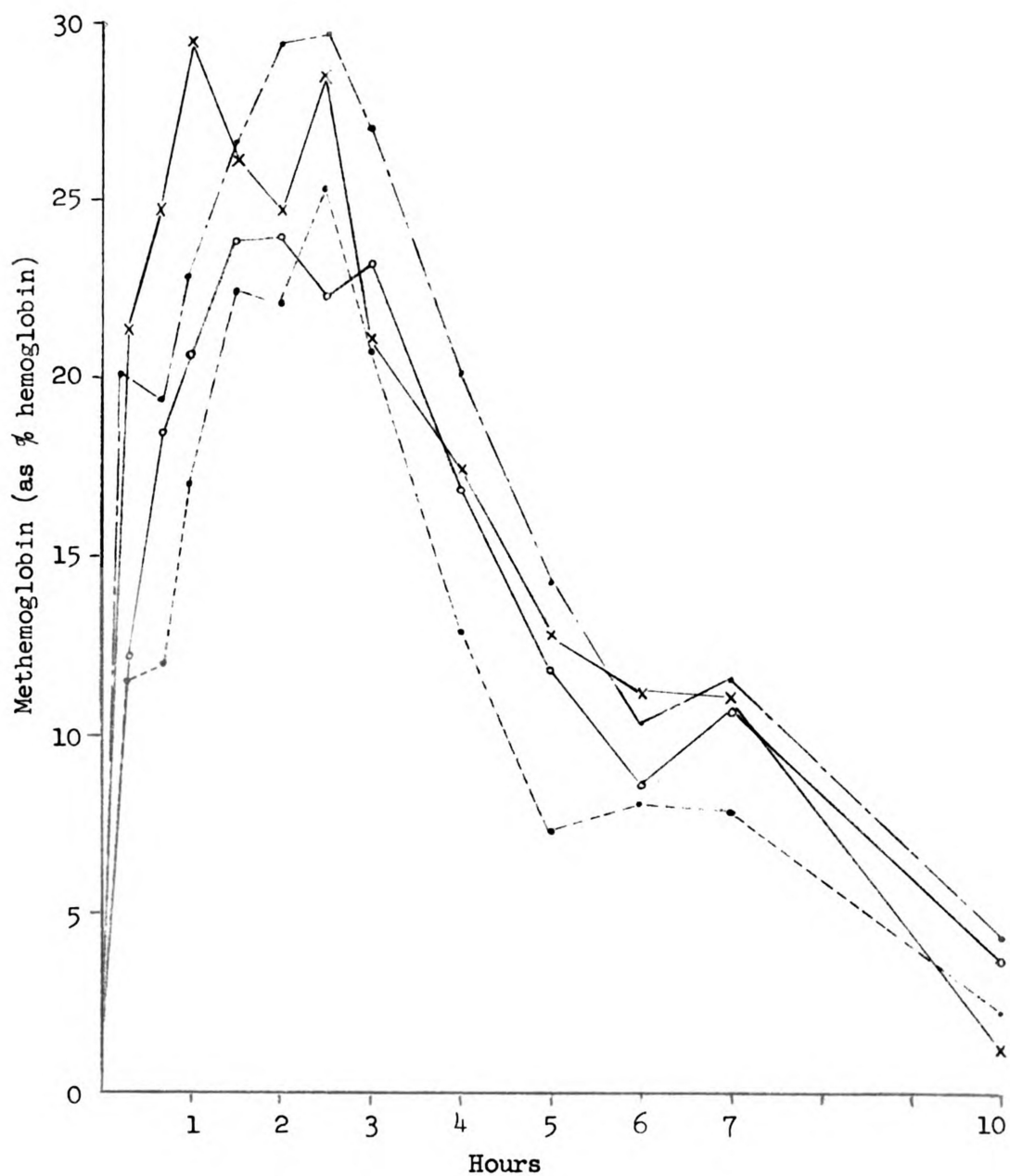


Figure 4. In vitro methemoglobinization of sheep blood induced by NaNO_2 10 mg./100 ml. blood. x—x sheep 35, •—• sheep 49, ○—○ sheep 173, —•— sheep 175.

Determinations of Nitrite in Lettuce After E. coli Reduction

Analysis of the lettuce after E. coli reduction revealed that there was not an appreciable amount of nitrite present. The mean nitrite value of 270 mg./100 Gm. dry weight represents approximately 1.7 per cent reduction of the nitrate originally present (Table 11).

Plasma Nitrate and Nitrite Results for All Blood Studies

Results were negative for all determinations for plasma nitrate and plasma nitrite during this experiment.

Determinations of Nitrite in Lettuce After E. coli Reduction

Analysis of the lettuce after E. coli reduction revealed that there was not an appreciable amount of nitrite present. The mean nitrite value of 270 mg./100 Gm. dry weight represents approximately 1.7 per cent reduction of the nitrate originally present (Table 11).

Plasma Nitrate and Nitrite Results for All Blood Studies

Results were negative for all determinations for plasma nitrate and plasma nitrite during this experiment.

Table 10. Methemoglobin values in sheep after oral administration of 275 mg. NaNO_2 /Kg. body weight one half hour after oral administration of Ru-Bac.

Animal		Methemoglobin (%) at Intervals							
No.	Wt.	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	7 hr.
19	39 Kg.	0.0	5.9	8.4	11.3	7.4	1.9	1.9	1.0
32	33 Kg.	1.3	2.4	3.8	2.4	4.8	6.8	5.8	4.7
165	50 Kg.	1.4	1.4	1.0	1.0	1.5	2.6	1.5	2.9
172	39 Kg.	1.3	5.4	12.3	15.7	5.8	5.8	0.0	0.0
Mean		1.0	3.8	6.4	7.6	4.9	4.3	2.3	2.3
S. D.		0.2	2.2	5.0	5.7	2.5	2.4	2.5	1.8

Table 11. Nitrite values of lettuce after E. coli reduction and original nitrate value before reduction.

Lettuce Administered to Sheep		Dosage mg. NO_2 /Kg. Body Wt.	Lettuce Dry Weight Gm.	Nitrate Microgm. Per Gm. Dry Wt.	Nitrite microgm. Per Gm. Dry Wt.
No.	Wt.				
19	44 Kg.	1.10	186	16,426	259
32	37 Kg.	1.66	186	16,426	325
49	41 Kg.	1.07	186	16,426	236

DISCUSSION

One of the manifestations of nitrite toxicity is the oxidation of hemoglobin to methemoglobin. The resulting methemoglobinemia is the major factor in the pathogenesis of nitrite toxicosis.

A public health problem exists due to current agricultural practices of heavy nitrate fertilization. The important factor is not so much that there is a lot of nitrate present but that plants are often subjected to conditions that allows the increased uptake of the nitrate. These conditions have been recognized for many years but it has been only recently established that the application of herbicides increases the uptake of nitrate in certain plant species. It was also determined that some commercially grown lettuce has a high nitrate content (21,000 to 24,000 ppm).

The nitrate in lettuce fed to sheep did not produce a rise in the circulating methemoglobin. Continued normal methemoglobin values were probably the result of (1) a relative small amount of nitrate being reduced to nitrite, (2) the released nitrite was converted to ammonia by the rumen bacteria or (3) the methemoglobin produced was reduced to hemoglobin as fast as it was being formed. The second hypothesis is supported by the observations of Lewis (1951) that nitrite was readily reduced to ammonia but that it did have a point at which nitrite began to accumulate in the rumen and was then absorbed into the blood. The third hypothesis is supported by the observations of Kitchen (personal communication) in that sheep blood readily reduces methemoglobin to hemoglobin. The method for giving the sheep large amounts of plant

material (as compared to fresh weight) proved successful. In this experiment an equivalent of 3.5 Kg. fresh weight lettuce was given as a paste made from the 182 Gm. of powdered lettuce.

The nitrate given at 150 and 275 mg. NaNO_3 /Kg. body weight did not produce a distinct rise in the circulating methemoglobin. The 400 to 600 mg. NaNO_3 /Kg. body weight levels of nitrate did produce a moderate rise in circulating methemoglobin. This is substantiated by Lewis (1951), that with less than 0.25 mole of nitrate (19 Gm. NaNO_3) a linear relationship exists between the amount of nitrate administered and the extent of methemoglobinemia. The greater quantities of nitrate result in a disproportionately large amount of circulating methemoglobin. This he attributed to the reaction of nitrite being reduced to form ammonia if the dosage is below 0.25 moles of nitrate. After the dosage exceeds 0.25 mole of nitrate the nitrite that is not converted to ammonia is absorbed into the blood stream and causes methemoglobinemia. This, Lewis, termed as the "limiting factor".

The nitrite studies were more clear cut as methemoglobin values were not as erratic as those in the nitrate studies. Nitrite given in the amount of 20 to 30 mg. NO_2 /Kg. body weight produced a mild to moderate methemoglobinemia. In the experiment in which 30 mg. NO_2 /Kg. body weight was administered there was 1 sheep that had a twofold higher methemoglobin value than the others. This can only be attributed to individual variation. Another individual variation was seen in the sheep given 40 mg. NO_2 /Kg. body weight, as 1 sheep had a moderate methemoglobinemia and the other 3 had a severe methemoglobinemia. The levels of nitrite-N (40 mg. NO_2 /Kg. body weight) required to produce moderate to severe methemoglobinemia in sheep was considerably lower than

that reported in 1951 by Lewis (115 mg. nitrite-N/Kg. body wt.) and in 1967 by Sinclair and Jones (60 mg. nitrite-N/Kg. body wt.).

The study of methemoglobinization of sheep blood by nitrite in vitro indicated that the variation in methemoglobin concentration seen in in vivo studies is due to variations in the rumen. The in vitro methemoglobin formation in blood samples from 4 sheep were all in close approximation to one another. This close approximation also occurred in the reduction of methemoglobin to hemoglobin.

There was only a minimal reduction of nitrate to nitrite in the lettuce treated with E. coli. The final analysis of the nitrite present in the lettuce indicated that only a 1.7 per cent reduction of the nitrate had occurred (Table 11). This resulted in a small dose of nitrite to the sheep which explains the lack of rise in methemoglobin. In working with the E. coli reduction method there was a concern about the nitrate being reduced to ammonia by the nitrite reductase activity found with these bacteria. A better procedure for reducing nitrate to nitrite without the presence of nitrite reductase may be to use bacterioid nitrate reductase. In this procedure a greater nitrate reduction to nitrite would result without loss of nitrite.

The addition of bacteria to the sheep rumen before giving NaNO_3 may have enhanced the reduction of nitrate to nitrite as shown in Table 10. However, in this experiment the observed difference was mainly due to 1 sheep. Apparently enough excess nitrite may be formed in a shorter period of time so that the limiting factor (Lewis, 1951) can be lowered. That is, the greater than normal amount of bacteria present in the rumen allows for a greater reduction of nitrate to nitrite in a given amount of time.

From this work one should not conclude that there is no potential toxicity in plants containing large amounts of nitrate. From a public health stand point further work in this area should be conducted. Suggested studies may include working with simple stomached animals, such as swine, with gastrointestinal infections. The addition of bacteria to the normal intestinal or rumen flora to enhance nitrate reduction may increase the toxicity. Additional work with herbicides in which higher levels of nitrate in plants are observed may necessitate further work to assess potential toxicity for animals and man.

SUMMARY AND CONCLUSIONS

This work was designed to determine if lettuce which contained relatively high concentrations of nitrate would elicit a methemoglobinemia in sheep. Basic to the accompanying characterization of the effect was a series of studies on the extent of methemoglobin formation caused by NaNO_2 in vitro and in vivo, and by NaNO_3 in vivo. Determinations of hemoglobin, methemoglobin formation, plasma nitrate and plasma nitrite were made. Hemoglobin values were also determined for each blood sample. From these experiments the following conclusions were made:

1. In the lettuce administered, the levels of nitrate (16,462 micrograms $\text{NO}_3/\text{Gm. dry wt.}$) did not produce a methemoglobinemia in sheep. Approximately 186 Gm. of powdered lettuce is equal to 3,370 Gm. of fresh lettuce. Thus, even in large quantities the nitrate in lettuce was not toxic.
2. In vitro E. coli reduction of nitrate to nitrite is not easily controlled. Possibly a better method would be to use nitrate reductase. Further investigation along this line could reveal better methods.
3. Sodium nitrate (600 mg./Kg. body wt.) or sodium nitrite (40 mg. $\text{NO}_2/\text{Kg. body wt.}$) orally administered results in mean methemoglobin concentrations corresponding to approximately 35 to 55 per cent conversion of the total hemoglobin, respectively.
4. After a moderate in vitro methemoglobinization (30 per cent), the blood enzyme system was capable of reducing methemoglobin to 95 per cent hemoglobin 7.5 hours after peak methemoglobinization.

5. In spite of some relatively high levels of methemoglobin, the hemoglobin values remained within their normal ranges in nitrate and nitrite treated sheep.

6. The level of nitrite (40 mg. NO_2 /Kg. body wt.) required to produce moderate to severe methemoglobinemia in sheep was considerably lower than previously reported (approximately 125 to 150 mg./Kg. body wt.).

7. The limiting factor of nitrate being reduced to ammonia before nitrite attains excess and becomes absorbed into the blood stream may possibly be lowered by the addition of bacteria to the rumen of sheep.

8. Sheep can be administered large amounts (2.5 to 3.5 Kg.) of plant material after dehydration of the plant, powdering and then making a paste from the powder. In sheep the most successful means of administering the paste was by a large (12 oz.) dose syringe.

REFERENCES

- Benjamin, M. M.: Outline of Veterinary Clinical Pathology, 2nd. ed.
The Iowa State University Press, Ames, Iowa, 1961.
- Bodansky, Oscar: Methemoglobinemia and methemoglobin-producing compounds. Pharmacol. Revs., Vol. 3, No. 2, 1951.
- Breniman, G. W., Neumann, A. L., Smith, G. S., Zimmerman, J. E.: Factors affecting the nitrate content of forages. J. Animal Sci., 20:926 (Abst.) 1961.
- Burden, E. H. W. J.: The toxicology of nitrates and nitrites with particular reference to the potability of water supplies. The Analyst, 86:420, 1961.
- Cohen, G., and Hochstein, P.: Generation of hydrogen peroxide by hemolytic agents. Biochemistry, 3:895, 1964.
- Comly, H. H.: Cyanosis in infants caused by nitrates in well water. J.A.M.A., 129:112-115, (Sept.3) 1945.
- Crawford, R. F., Kennedy, W. K., and Wright, M. J.: Nitrate in forage crops and silage. Bulletin 37, New York State College of Agriculture, 1960.
- Davison, K. L., Hansel, W., Wright, M. J., and McEntee, K.: Adaptation to high nitrate intake by cattle. Proceeding for 1962 of the Cornell Nutrition Conference for Feed Manufacturers.
- Davison, K. L.: Sublethal nitrate poisoning - is it really a problem? Feed Age, 23-27, (March) 1966.
- Diven, R. H., Pistor, W. J., Reed, R. E., Trautman, R. J., and Watts, R. E.: The determination of serum or plasma nitrate and nitrite. Am. J. Vet. Res., 23:497-499, 1962.
- Emerick, R. J., Embry, L. B., Surley, R. W.: Rate of formation and reduction of nitrite-induced methemoglobin in vitro and in vivo as influenced by diet of sheep and age of swine. Journal of Animal Sci., 24:221-228, (Feb.) 1965.
- Evelyn, K. A., and Malloy, H. T.: Microdeterminations of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem., 126:655-662, 1938.
- Fassett, E. W.: Toxicants occurring naturally in foods. Nitrates and nitrites. National Academy of Science - National Research Council Publication, 1354:250-256, 1966.

- Hageman, R. W., Cresswell, C. F., and Hewitt, E. J.: Reduction of nitrate, nitrite, and hydroxylamine to ammonia by enzymes extracted from higher plants. *Nature*, 193:247, 1962.
- Hölscher, P. M. and Natzschka, J.: Methaemoglobinaemia in young infants due to nitrite in spinach. *Germ. Med. Mon.*, 9:325-327, 1964.
- Keohane, K. W. and Metcalf, W. K.: An investigation of the sensitivity of juvenile and adult erythrocytes to methaemoglobinization. *Physics in Medicine and Biology*, 5:27-35, 1960.
- Kiese, M.: The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. *Pharmacol. Rev.*, 18:283-290, 1966.
- Lewis, D.: The metabolism of nitrate and nitrite in the sheep. I. The reduction of nitrate in the rumen of the sheep. *Biochem. J.*, 48:175-180, 1948.
- Metcalf, W. K.: A biochemical change in the blood in pregnancy and malignant disease. *Phys. Med. Biol.*, 5:259-268, 1961.
- Metcalf, W. K.: The sensitivity of intracorpuseular hemoglobin to oxidation by nitrite ions. I. The effect of growth, starvation and diet. *Phys. Med. Biol.*, 6:427-435, 1962.
- Orgeron, J. D., Martin, J. D., Caraway, C. T., Martine, R. M., and Hauser, G. H.: Methemoglobinemia from eating meat with high nitrite content. *U. S. Public Health Rept.*, 72:189, 1957.
- Phillips, W. E. J.: Changes in the nitrate and nitrite contents of fresh and processed spinach during storage. *J. Agr. Food Chem.*, Vol. 16, No. 1, 88-91, (Jan.-Feb.) 1968.
- Ries, S. K. and Gast, A.: The effect of simazine on nitrogenous components of corn. *Weeds*, 13:272-274, 1965.
- Ries, S. K.: The increase in protein content and yield of simazine-treated crops in Michigan and Costa Rica. *Bio Science*, Vol. 18, No. 3, 205-208, 1968.
- Ross, J. D.: Deficient Activity of DPNH-dependent methemoglobin idaphorase in cord blood erythrocytes. *Blood*, 21:51, 1963.
- Setchell, B. P. and Williams, A. J.: Plasma nitrate and nitrite concentration in chronic and acute nitrate poisoning in sheep. *Aust. Vet. J.*, 38:58-62, 1962.
- Simon, J., Sund, J. M., Wright, M. J., Winter, A., and Douglas, F. D.: Pathological changes associated with the lowland abortion syndrome in Wisconsin. *J.A.V.M.A.*, 132:164-169, 1958.

- Simon, J., Sund, J. M., Douglas, F. D., Wright, M. J., and Kowalczyk, T.: The effect of nitrate or nitrite when placed in the rumen of pregnant dairy cattle. J.A.V.M.A., 135:311-341, 1959.
- Sinha, D. P.: Pathogenesis of abortion in acute nitrite toxicosis in guinea pigs. Ph.D. Thesis, Michigan State University, 1968.
- Sleight, S. D. and Sinha, D. P.: Prevention of methemoglobin reduction in blood samples. J.A.V.M.A., 152:1521-1525, 1968.
- Spicer, S. S.: Species differences in susceptibility to methemoglobin formation. J. Pharmacol. Exp. Therap., 99:185-194, 1966.
- Stolk, J. M. and Smith, R. P.: Species differences in methemoglobin reductase activity. Biochem. Pharmacol., 15:342-351, 1966.
- United States Public Health Service Drinking Water Standards: U. S. Public Health Service Publication, 47, 1962.
- Winter, A. J.: Studies on nitrate metabolism in cattle. Am. J. Vet. Res., 23:500-505, 1962.

VITA

Robert Lee Amster was born in Madison County, Kentucky on October 12, 1934. He graduated from Lafayette High School, Lexington, Kentucky in 1952.

In 1955, after service in the United States Navy, he enrolled at the University of Kentucky and in September 1958 transferred to Auburn University, Auburn, Alabama and there received his degree of Doctor of Veterinary Medicine in 1962.

From 1963 to 1966 the author was employed by Florida Department of Agriculture Diagnostic Laboratory in Kissimmee, Florida. While there he co-authored two papers, "Studies of the Lipoprotein Methods Used for Detecting Altered Serum Lipoproteins in Equine Infectious Anemia" and "Altered Serum Lipoproteins in Equine Infectious Anemia: Comparisons of Values Among Normal Horses and Horses Infected with *Babesia caballi*".

In October 1966, the author received a commission as Captain in the United States Air Force, and has served active duty at Wilford Hall Hospital, Lackland Air Force Base, Texas and at Battelle Memorial Institute Pacific Northwest Laboratory, Richland, Washington.

After completing schooling at Michigan State University, he will continue his studies at the Armed Forces Institute of Pathology, Washington D. C.

Dr. Amster is married to the former Mary Frances Hall and is the father of three children; Robert Koerner, Deborah Gale, and Brenda Leigh.

MICHIGAN STATE UNIV. LIBRARIES



31293000842595