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# Copper, Molybdenum, and Sulphur Interactions in Ruminant Nutrition

by

© Peter J. Vitti

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As I entered the graduate program in Animal Science nearly four years ago, I failed to recognize the challenges which laid ahead. I later discovered that not only was my knowledge tested but my imagination was drawn upon for creating and conducting three special cattle projects. As a result of these experiments I hope I have contributed to building a better beef industry.

As his graduate student, my first thanks goes to Dr. T.J. Devlin for giving me the independence to execute such fine projects but at the same time helping me in solving experimental problems which did arise. I also thank Dr. G.D. Phillips for the surgical work of the projects. Mr. J.A. McKirdy and Clif Batenchuk were also extremely helpful in the mineral analysis of the biological and feed samples. My special thanks goes to Millen Johnson, Bob Lavallee, Mike Stuski and Lorne Dawydiuk and Phil Stoess for feeding and maintenance of the beef animals used in the projects, and assistance for acquiring biological samples. I am also indebted to Ben Haakman and Jerry Levandoski for their preparation of the animal diets used in the experiment.

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## ABSTRACT

Three experiments were conducted to study various aspects of copper-molybdenum-sulfur interactions in ruminant nutrition.

In the first experiment, the effects of different concentrations of dietary copper (Cu) and molybdenum (Mo), in a 2:1 ratio, on copper status of beef yearling steers were studied.

Sixteen yearling beef steers weighing about 360 kg each were divided into four treatment groups. The treatment groups were fed one of four diets for a total 121 days. Each group received approximately 36 kg (9 kg per animal) of assigned treatment diet daily.

The basal diet consisted of three ingredients, namely: 42.5% alfalfa-bromegrass hay, 55% barley and 2.5% mineral and urea premix. Copper oxide (75% Cu) and ammonium molybdate (57.7% Mo) were supplemented in the premix in order to provide the various concentrations of Cu and Mo in a 2:1 ratio in the diet containing basal levels of 5.79 ppm Cu and 1.11 ppm Mo. The entire diet was fed to the cattle in pelleted form. The treatment groups were as follows: (1) 7.0 ppm Cu:3.5 ppm Mo, (2) 15 ppm Cu:7.5 ppm Mo, (3) 30 ppm Cu:15 ppm Mo, (4) 100 ppm Cu:50 ppm Mo.

Statistically, the experiment followed a split-plot design in which several criteria were utilized to measure copper status of the steers during the experiment. These parameters included liver Cu, total serum Cu, TCA-soluble Cu and ceruloplasmin (a Cu dependent enzyme) activity. All animals were weighed and liver biopsies obtained approximately every 30 days, while jugular blood samples (for serum) were obtained weekly.

Live weight gain, ADG and dressed carcass weight of the steers did

not differ significantly ($P>.05$) among treatment groups. Liver Cu levels of the steers were significantly ($P<.05$) affected by treatment and were also significantly ($P<.01$) increased by period. The levels of total serum Cu of intervening periods as well as those of the other serum Cu parameters were not affected ($P>.05$) by treatment although they tended to increase with time on treatment.

Second and third experiments were conducted to examine the effects of dietary sulphur (S) upon copper status of beef yearling steers.

The second experiment followed a balanced 4x4 latin square in which each of four treatment diets were fed (9 kg/day per animal) to four steers in four 28-day treatment periods. There was a fourteen day rest period between each treatment period in which all four animals were fed the same diet.

The treatment diets (pelleted) consisted of a basal ration of 45% barley, 52.5% alfalfa-bromegrass hay and 2.5% mineral and urea premix. Sodium sulphate was added to the premix to provide a final concentration of 0.2%, 0.3%, 0.4% and 0.5% S in the treatment diets. The resting diet was identical to the 0.2% S treatment diet. Copper oxide and ammonium molybdate were added to the premix to raise the basal levels of Cu and Mo to 20 ppm Cu and 5 ppm Mo (4:1 ratio), respectively.

Feed consumption was measured daily, and all animals were weighed and liver biopsies taken every six weeks with jugular blood being sampled weekly.

The amount of feed consumed did not differ significantly ($P>.05$). among treatment diets or among steers. Trace mineral analysis of samples

indicated that liver Cu levels of steers were not significantly ($P>.05$) affected by treatment but differed significantly ($P<.01$) among periods and were significantly ($P<.05$) different among steers. Serum Cu and TCA-soluble Cu levels were not significantly ($P>.05$) affected by steer, treatment or periods while ceruloplasmin activity was significantly ($P<.01$) altered by both steer and period.

In the third experiment, sixteen steers weighing an average of 306 kg were divided into four treatment groups. Each group was assigned to one of four S-treatment diets: 0.2%, 0.3%, 0.4% and 0.5% for 118 days. The formulation of these diets was virtually identical to diets fed in Experiment 2, however 3.0% molasses was incorporated into the present diets. Each group received 36 kg of pelleted diet daily.

The third experiment followed a split-plot design in which liver Cu, total serum Cu, TCA-soluble Cu and ceruloplasmin activity were measured to establish the copper status of the steers. Feed consumption among pens was measured on a daily basis whereas all animals were weighed and liver biopsies obtained every 30 days and jugular blood sampled weekly.

Feed consumption did not differ significantly ($P>.05$) among treatment groups. Live weight gain, ADG and dressed carcass weight were also not significantly ($P>.05$) affected by treatment.

Liver Cu of the steers was not significantly ($P>.05$) affected by treatment; however, copper accumulated in the livers ($P<.01$) over time. Total serum Cu and TCA-soluble Cu levels were not ($P>.05$) affected by sulphur treatments but varied significantly ($P<.01$) for the intermediate samples taken during the experiment. Serum ceruloplasmin activity levels

were significantly ($P<.05$) different among treatment groups and there were also significant ($P<.01$) period effects. Serum Cu levels were highly correlated with both TCA-soluble Cu and ceruloplasmin activity levels ($r = .84$, $r = .75$, respectively).

TABLE OF CONTENTS

Page

# LIST OF TABLES

# LIST OF FIGURES

Figure | Page

# LIST OF APPENDIX TABLES

Appendix 1: Experiment 1

Appendix I: Page

Appendix II: Page



Appendix III: Experiment 3

Appendix III:

# REVIEW OF LITERATURE

## Introduction

Lack of energy and protein in a diet are often deemed responsible for the poor performance of many livestock herds. Although a shortage of these two essential components may upset optimum production, many cattle are still under-performers in spite of an abundant supply of feed containing adequate energy and protein. Recently, much information is being discovered linking these latter cases with mineral deficiencies and/or imbalances. Even though many minerals are needed in very small quantities, namely, trace minerals, they play a very essential role in the vital processes of the body. The deficiency or imbalance of a trace mineral in a diet that may be otherwise adequate can limit livestock growth and development as dramatically as a diet inadequate in energy or protein.

Mineral imbalances in ruminants are not uncommon and many incidences of trace mineral deficiencies are recorded throughout the world. Grazing cattle in tropical locations (Asia, Africa, Latin America) normally do not receive any mineral supplementation. Since the animals rely on forages from mineral poor soils, the animals understandably might not meet their basic mineral requirements (McDowell et al. 1983). Latin American forages are frequently borderline or deficient in elements such as cobalt, copper, magnesium, phosphorus, sodium and zinc (McDowell 1983).

Copper is one of many trace minerals necessary for the maintenance and optimum productivity of ruminants. Since the late 1920's, copper

had been considered to be an essential nutrient. In 1928, Hart and his coworkers found dietary copper played an essential role in hemoglobin synthesis in rats (Bull 1980). A few years later, copper supplements were shown to alleviate or cure many apparent disease conditions common to grazing ruminants all over the world.

Deficiencies of copper in ruminants are a result of the animals failure to acquire enough copper for body needs. NRC (1976) states that the requirement of cattle for copper is 4 mg/kg dry feed. Failure to provide this level of copper in the cattle diet would result in symptoms of a primary copper deficiency. Many sources reveal that 10 mg/kg Cu in a cattle diet may be necessary to compensate for antagonistic compounds that reduce biological availability of copper in the animal (Boila et al. 1984a). Many studies have demonstrated that a diet containing a marginal level of copper in combination with high levels of dietary molybdenum (greater than 3 mg/kg) can induce a secondary or conditioned copper deficiency. In addition, above normal levels of sulphate (greater than .3%) in the diet may potentiate the adverse effects of molybdenum upon copper status in the animal (Boila et al. 1984a).

Although a general requirement for copper is recommended to prevent a primary copper deficiency and to counteract the antagonistic actions of Mo and S, a well defined copper requirement for cattle has not been established. Because of many nutritional factors affecting copper metabolism, a specific requirement for ruminants often can not be assigned. In addition to nutritional factors, there are many non-nutritional elements which direct the actions of copper in the body.

More research is being conducted concerning the presence and function of copper in the ruminant. Criteria for determining copper status in cattle are consistently being scrutinized for variability and reliability. Furthermore, much information is coming to light focussing upon the interrelationship among copper, molybdenum and sulphur and its effects on established copper parameters. Biochemical mechanisms explaining how such minerals interact are still unclear, but more studies have established working models of Cu-Mo-S interactions.

In order to better understand the role of copper in ruminant nutrition different aspects of this trace mineral will be examined. Copper is distributed throughout the body and concentrated in specific organs. Copper also plays a key function in specific metalloproteins and enzyme systems. The determination of copper status as well as importance of copper in metalloproteins and enzyme systems will be discussed. As with other trace minerals, copper is absorbed into, transported within, stored in and excreted from the body. An examination of the mechanisms involved in copper metabolism will be presented with special emphasis focussed upon copper homeostasis in the ruminant. Primary and secondary or conditioned copper deficiencies in cattle occur under low dietary copper situations or as a result of high levels of dietary molybdenum and/or sulphur. The interactions between respective pairs of these elements will be examined, with major attention given to how such relationships modify copper status in the ruminant. Finally, special recognition will be given to the thiomolybdate theory. This hypothesis, based upon research, attempts to explain the combined antagonistic effects of molybdenum and sulphur upon copper metabolism in ruminants.

Absorptive and post-absorptive mechanisms that reveal the existence of a Cu-Mo-S complex will be examined.

## Copper Distribution

Copper is distributed throughout the body and is concentrated in certain organs (Evans 1973). In general, the liver, brain, heart and kidney in decreasing order, contain the highest concentrations of copper, however the greatest amounts of copper on an absolute basis are found in the muscle and bone. Although these two tissues contain the lowest concentrations of copper in the body, due to their large mass, muscle and bone contain about 50% of total body copper while the liver contains only about 10% of total body Cu (Evans 1973). The average copper concentration in the body is only about 1.5-2.5 mg/kg in the adult animal (Bull 1980), and varies with species and age of the animal, diet, disease and pregnancy (Underwood 1977).

The liver has long been recognized as the major organ of copper accumulation in the body. Under a normal dietary feed regime of copper (approximately 10 mg/kg diet) the liver contains 25-150 mg/kg (wet basis) in cattle (Puls 1981). However the distribution of this copper varies within an individual liver. O'Cuill et al. (1970) acquired 13 bovine livers and randomly sampled each liver in six different areas for copper analysis. The results showed a wide variation in copper distribution in the examined bovine livers. While the range of liver copper concentration in a certain animal was quite small (13-19 mg/kg DM), another subject revealed a wide range among the six areas of about 100 mg/kg DM (29-123 mg/kg DM).

In a similar experiment, Bingley and Dufty (1972) reported that distribution of copper within the livers of 4 neonate calves was uneven. Within the main body of the liver, the copper concentration of biopsy samples was highest in the lateral half of the dorsal lobe. In each calf, the highest copper concentration was found in the caudate lobe. Furthermore, the concentration of hepatic Cu at points along the tenth intercostal space also varied. Chapman et al. (1963) also discovered that the level of copper among the livers of beef steers was dependent on the individual liver and the biopsy site. They acknowledged that liver Cu content of cattle in a normal copper state can range from 150 to 250 mg/kg DM whereas copper deficient cattle usually have 10-25 mg/kg in hepatic copper. However, Chapman et al. (1963) stated that concentration differences among biopsy sites, though statistically significant were relatively small when compared to ranges of Cu found for the livers of copper-normal and copper-deficient steers.

Regardless of Cu distribution in the liver, the concentration of copper stored in the liver is affected by many factors. For example, a newborn calf contains a higher concentration of liver copper than its dam. Bingley and Dufty (1972) compared the copper concentration of the liver and various organs of 4 newborn Hereford calves and 2 cows which had grazed pasture normal in copper (8 to 9 mg/kg DM). All four calves were produced from these 2 cows. Trace mineral analysis of the neonate livers revealed a range of 195-715 mg/kg, DM copper, compared to 32.7 and 38.2 mg/kg Cu in the two dams respectively. Aside from the liver, only the skin, tongue, bone and tunica of the eye were significantly

higher in copper in the bovine neonate. Evans (1973) explained that the significantly higher hepatic copper level in the neonate animal, when compared to the adult, is a result of lack of development in the neonate of the copper excretory mechanism that is regulated by the pituitary-adrenal endocrine system. He stated that in the case of the rat, the pituitary endocrine system is not fully activated until the 3rd week of life. At this time, the rat subsequently began to mobilize hepatic copper.

In the liver, copper is distributed among hepatic subcellular fractions in association with specific copper binding proteins and copper dependent enzymes such as superoxide dismutase (Evans 1973). The cytosol fraction of the hepatic cell contains the major portion of hepatic copper. A large portion of cytosol copper is bound to specific metal binding proteins called metallothioneins. Metallothionein, a specific low molecular weight protein (MW-10,000), known for its extremely high affinity for metals, which include Cu and Zn is the focus of transportation, storage and detoxication of these elements (Bremner and Marshall 1974a, b). The occurrence and metal content of these specialized proteins are dependent on Cu and Zn status of the animals.

A hepatic protein sub-fraction containing more than 4% copper has been isolated from the mitochondrial portion of the neonate calf (Underwood 1977). Similar amounts of this copper binding protein are not present in adult bovine livers. Evans (1973) identified this copper-binding protein as neonatal hepatic mitochondrocuprein. He also pointed out that this Cu-protein is small (MW-5,000-10,000). Mitochondrocuprein is believed to function as a copper reserve for the synthesis of cyto-

chrome oxidase during neonatal development. Underwood (1977) further pointed out that neonatal hepatic mitochondrocuprein has a very high cysteine content (about 35%) and a similar amino acid composition to metallothionein. Furthermore, Underwood (1977) suggested that copper thioneins are possibly the low molecular weight form of neonatal hepatic mitochondrocuprein. Bingley and Dufty (1972) found that copper in the caudate lobe of the neonatal liver, as well as the dorsal or ventral lobes remained unstained by a histological stain, rubeanic acid. These results suggest copper was bound tightly to mitochondrocuprein, since less tightly bound forms of copper are easily stained by rubeanic acid.

In the blood, copper is a versatile component of many metalloproteins and metalloenzyme systems. Copper has many special biochemical properties which enable it to be an effective biological binding agent. Friedan (1968) reported that copper can exist in an array of ionic states that direct its metabolic functions. He also pointed out that one ion can easily convert to a different form, simply by adding or releasing an electron which converts copper into an electron acceptor or an electron donor. Such flexibility in electron states allows copper to be an effective oxidative catalyst (i.e. ceruloplasmin/ferroxidase). Friedan (1968) also stated that copper ions bind with amino acids and protein more tightly than other metals and form very stable chelates with biologically active substances. Such associations can only be broken by drastic chemical means.

In the red blood cell, approximately 60% of the copper occurs in the colorless protein, erythrocuprein (hemocuprein). Erythrocuprein contains about 0.34% Cu and has a molecular weight of about 35,000

(Underwood 1977). Previous research isolated a number of erythrocuprein-like copper proteins from bovine erythrocytes and livers as well as from human erythrocytes, brain and liver (Evans 1973). Each protein was given its own nomenclature and at first they were thought to be unrelated. Further experiments showed that all these proteins contain similar amounts of copper and were of similar molecular weight. Recently it was proven that all these proteins were identical (Evans 1973).

Erythrocupreins are now classified as superoxide dismutase. The protein has the enzymatic ability to catalyze the dismutation of monovalent superoxide anion radicals to hydrogen peroxide and oxygen (Underwood 1977). The reaction is as follows: $O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$. Superoxide dismutase in the red blood cell and in other sites protect the cell from damage caused by formation of free radicals in the body.

In the plasma, Cu is present in three forms: Cu in ceruloplasmin (80-90%), Cu loosely bound to albumin (15-20%) and copper bound to amino acids (2-3%) (Ischida et al. 1982). Although ceruloplasmin normally accounts for more than 90% of the copper in the plasma, ceruloplasmin also makes up about 3% of the total copper in the whole body (Amer et al. 1973). In greater detail, ceruloplasmin is classified as a blue alpha-globulin ($\alpha$-glycoprotein) with a molecular weight of 151,000 and contains about .34% copper (Rice 1963). Every molecule of ceruloplasmin contains 8 firmly bound atoms of copper. Cu can be released from the ceruloplasmin apoprotein by treating the plasma with trichloracetic acid (TCA). This TCA-soluble fraction is designated as the soluble portion of the plasma,

containing the Cu from ceruloplasmin and from albumin (Mason et al. 1978).

Much information has demonstrated that ceruloplasmin functions as a ferroxidase, involved in iron utilization and in promoting iron saturation of transferrin in the plasma (Underwood 1977). Ceruloplasmin has very little to do with Cu transport from absorption sites of the gastrointestinal tract to the liver and from the liver to peripheral tissues. This aspect of copper metabolism is executed by the albumin, which loosely binds copper. In vitro, it has been shown that ceruloplasmin exerts its biochemical properties by oxidizing p-phenylenediamine (PPD) (Amer et al. 1973). In this system the enzymatic activity of ceruloplasmin is proportional to its copper content. In addition, substances (e.g. sodium azide) that inhibit other copper dependent enzymes will retard ceruloplasmin action (Amer et al. 1973).

More recently, a fourth transitory fraction of Cu in plasma has been shown to exist which contains metabolically unavailable Cu in a Cu-Mo-S complex (Mason et al. 1978). Ischida et al. (1982) found that the addition of molybdenum and sulphate to the diets of sheep increased the biologically unavailable form of copper while decreasing TCA-soluble Cu (ceruloplasmin and albumin-Cu). These authors concluded that this copper represents a more tightly bound form of copper, than that in ceruloplasmin and appears to be unavailable for utilization by the animal.

Other Cu-dependent enzymes in the body include the amine oxidases (DiSilvestro and Cousins 1983). One particular amino oxidase, lysyl oxidase is involved in the formation of crosslinkings in collagen and

elastin fibers, necessary for connective tissue and bone formation. Copper also has been implicated to play an essential role in ferrochelatase activity (Maclean 1978). A ferrochelatase enzyme is responsible for the connection of a ferrous iron atom to a precursor heme component to yield a finished heme molecule. Likewise, Cu-containing cytochrome oxidase plays an important role as a terminal oxidase in the electron-transport chain of the mitochondria (Evans 1973). Cytochrome oxidase catalyzes the reaction between molecular oxygen and reduced cytochrome C. Subsequently, the oxygen is reduced to water. Therefore, severe copper deficiency is thought to impair cellular respiration. Other known Cu-containing enzymes include tryptophan-2, 3 dioxygenase, mitochondrial monoamine oxidase and butyryl coenzyme A dehydrogenase (DiSilvestro and Cousins 1983).

## Measuring Copper Status

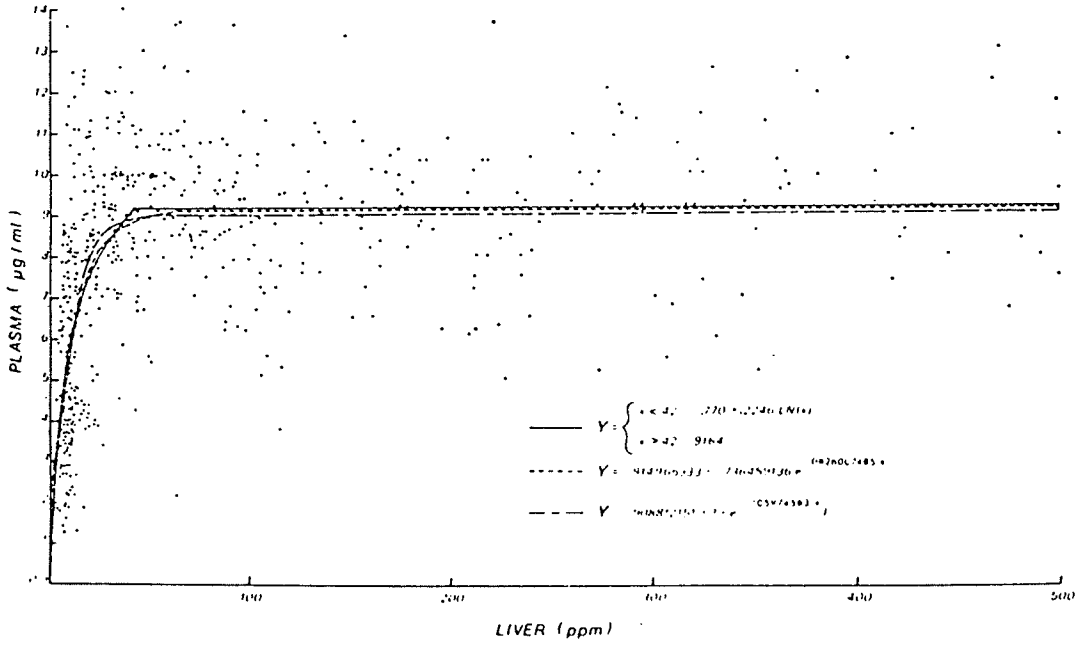
In the most severe cases of copper deficiency, ruminants exhibit clinical signs associated with a copper responsive disorder. However, few trace mineral deficiencies produce specific symptoms (Suttle 1976). There may be many alternative explanations for the gross symptoms associated with a copper deficiency. A particular symptom can be attributed to (i) a Cu-deficiency, (ii) simultaneous deficiencies of other elements (i.e. cobalt) and (iii) general undernutrition or to non-nutritional factors (i.e. parasitic infections) (Suttle and Angus 1976). The choice of biochemical parameters (i.e. plasma-Cu, liver-Cu, Cu-enzymes) to measure copper status in ruminants is complicated (Suttle 1976). This is because of the poor correlation of such copper parameters among themselves and with the clinical copper condition. Thus the best

indicator of copper status in ruminants is highly controversial (Mills et al. 1976).

Blood or plasma copper is widely accepted for determining copper status in ruminants. Givens and Hopkins (1981) demonstrated that cattle injected with a copper preparation were responsive in terms of plasma copper levels. The treatment either increased the plasma level to normal (between 0.70 and 1.1 µg/ml) in animals which began with low Cu values or Cu plasma levels remained unchanged in cattle commencing the study with adequate Cu plasma values. Engel et al. (1964) obtained blood as well as liver samples from Holstein calves fed four designated treatments. They found that calves on an unsupplemented Cu ration (4 to 5 ppm Cu) had significantly lower liver copper levels, while no ration effect was found for copper levels in the whole blood. Although, liver and blood copper parameters were significantly correlated ($r = .57$), it was concluded that the liver concentrations more closely reflected copper levels in the ration.

Bingley and Anderson (1972) acknowledged that copper deficient states in livestock were previously determined only by blood copper concentration but more information has shown that blood copper levels are maintained at the expense of liver copper reserves. From the Cu analysis of blood and liver samples from 540 cattle, Claypool et al. (1975) studied the relationship between plasma copper and liver copper. Generated by non-linear and linear least squares, a best fit curve was established between the two copper parameters (Fig. 1). The authors concluded that plasma copper was not a good indicator of copper status in ruminants unless plasma levels were below 0.5 µg/ml.

Figure 1. Relationship between blood plasma copper and liver copper and best fit curves generated by linear and nonlinear procedures. Claypool et al. (1975)



Determining a clinical copper deficiency by analysis of copper in the liver also has its disadvantages because copper concentrations in the liver are highly variable at different biopsy sites. Furthermore, a farmer may refuse to let his prize animals undergo the liver biopsy procedure (Dreosti and Quicke 1968).

In many studies, ceruloplasmin analysis has been preferred over total serum Cu as an indicator of copper status in ruminants. Since 80-90% of the serum/plasma copper is normally found in ceruloplasmin, one can expect a high correlation between ceruloplasmin and serum or plasma copper levels. Correlation coefficients ranging from .80-.94 between whole blood Cu and serum ceruloplasmin are not uncommon (Todd 1970). Copper is an integral part of the ceruloplasmin molecule and its concentration in the serum is directly proportional to corresponding serum or whole blood Cu levels (Thompson and Todd 1976). Todd (1970) surveyed the cattle on several farms in Northern Ireland using ceruloplasmin as a rapid method for detecting copper deficiency. A later study by Thompson and Todd (1976) comprised a more extensive survey of 20,000 cows on 1,200 farms, again using ceruloplasmin determination to identify Cu-deficient herds in Northern Ireland.

From both surveys, the advantages of using ceruloplasmin over blood Cu levels were recognized. Firstly, there is a low contamination risk associated with the ceruloplasmin enzyme assay. In contrast, trace mineral analysis requires a high standard of cleanliness at all stages from collection to determination of Cu (Todd 1970). Secondly, a small serum sample is required for ceruloplasmin analysis and the enzyme assay itself is a relatively rapid procedure (Lorentz and Gibbs 1975).

The analysis of ceruloplasmin activity in blood plasma from the ruminant does have its limitations. If the plasma is hemolyzed, variable results in ceruloplasmin activity may be recorded (Lorentz and Gibbs 1975). Also, many ceruloplasmin studies express serum ceruloplasmin activity in terms of arbitrary units based on the conditions of a particular procedure making between study comparisons very difficult. However, there are methods to standardize such ceruloplasmin results with the International Enzyme Units (Rice 1962).

Many researchers have produced an array of data resulting from feeding molybdate, sulphate or thiomolybdate to sheep. Ischida et al. (1982) demonstrated that dietary molybdenum in combination with added sulphur, significantly altered the distribution of copper in the blood. The sheep fed the Mo-$SO_4$ diet had an increase in direct-reacting Cu and a decrease in TCA-soluble copper and ceruloplasmin activity. On the other hand, Lamand et al. (1980) detected a sharp rise in TCA-soluble Cu and total plasma Cu in sheep fed a diet supplemented with S and Mo. From these sulfur-molybdenum studies, the distribution of copper in the blood based on TCA-solubility factors is a better indicator of copper status than total plasma copper in the ruminant (Mason et al. 1980).

The general drawback of many current copper criteria to establish copper status in ruminants is the poor correlation between the criteria and clinical condition. Suttle (1983) reported that the conventional copper parameters, liver and plasma copper, fall to below accepted normal levels during copper depletion in sheep and cattle while the animal may appear to be clinically normal. Suttle (1976) demonstrated the relationship between blood and liver parameters and the onset of clinical

conditions for trace mineral depletion. Chronologically, depletion commences once the animal fails to meet its own trace mineral requirement. Firstly, there is a decrease in non-essential reserves of the element in storage organs (i.e. liver). Secondly, a decline in the part of non-essential amounts at other sites (i.e. plasma) follows. Consequently, sub-clinical symptoms may occur. Finally, abnormal clinical symptoms appear once essential forms of the element disappear from key functional sites.

In accordance with the schematic plan, Suttle (1976) acknowledges that liver Cu often drastically falls before plasma Cu shows any change. Furthermore, ruminants which are classified as hypocupraemic are often regarded as clinically normal.

Suttle (1983) stated that a copper criterion is needed that would change less rapidly in response to depletion and then be more closely correlated with clinical and sub-clinical copper deficiencies. It was suggested that changes in activity of super-oxide dismutase in the erythrocytes (ESOD) may be a better indicator of copper status in ruminants. Suttle (1983) demonstrated that ESOD activity responded significantly more slowly than plasma copper in copper depletion and repletion situations in lambs. The lag time between changes in ESOD activity and plasma copper level was particularly evident in severely depleted lambs.

The disadvantage noted in using the new criterion for determinations of copper status in ruminants (Suttle 1983) was that ESOD responded too slowly in situations where high levels of dietary molybdenum (more than 10 mg/kg) rapidly induced copper deficient symptoms. In these cases,

the more conventional criteria for measuring copper status in ruminants, namely liver and plasma copper levels were more indicative of copper status.

## Copper Metabolism-Absorption

The site of copper uptake by the digestive system is species dependent although in most animals copper is absorbed from the duodenum and jejunum. Sheep, however, absorb a large portion of their copper from the large intestine (Underwood 1977). The absorption of copper from the small intestine is affected by several factors including the copper status of the animal (Bremner and Mills 1981).

Copper absorption in the small intestine is a two step process (Evans 1973). First copper is taken up by the mucosal cells and secondly transferred across the mucosal cells to the serum where it is linked to a Cu-transporting albumin (Fischer et al. 1981). DiSilvestro and Cousins (1983) found that copper crosses the mucosa bound to certain amino acids. They cited previous research which demonstrated that copper uptake was greater in animals fed copper-amino acid complexes than those animals fed equivalent amounts of copper as $CuSO_4$. L-amino acids (i.e. L-histidine) were also found to be more effective than D-isomers. Such an amino acid complex crossing the intestinal mucosa is thought to be an energy dependent process (Evans 1973).

There are many factors which control the amount of copper absorbed by the intestinal mucosa. A feedstuff may contain an absolute amount of copper necessary to meet the ruminant's requirement; however only part of this copper may be biologically available (Rusoff 1981). In

general, only about 1% to 3% of dietary copper in a feedstuff is absorbed in the ruminant (Hansard 1983). The extent to which copper is utilized depends on the form of copper present in the feedstuff. From radioactive tracer studies Underwood (1977) ranked several inorganic preparations for bioavailability in beef cattle. The relative appearance of $^{64}Cu$ in the blood was in the following order: $CuCO_3$>$Cu(NO_3)_2$>$CuSO_4$>$CuCl_2$>$Cu_2O$> Cu oxide (powder)>CuO (needles)>Cu (wire).

Conditions in the gastrointestinal tract are not always optimal for copper absorption. Likewise many dietary components may affect copper absorption by the small intestine. Solubility, molecular mass and cationic or anionic state of Cu-ligand complexes are significantly altered by the pH gradient of the gut (Bremner and Mills 1981). Acidic conditions will prevent the release of copper from particulate fractions of digesta and will reduce soluble copper concentrations, thereby reducing copper absorption. High fiber diets provide Cu-binding ligands that result in low absorption by the small intestine (DiSilvestro and Cousins 1983). Phytic and ascorbic acid will readily bind copper to form stable complexes and render copper unavailable for absorption (Underwood 1977). Bile also contains substances which prevent the reabsorption of excreted copper, in order to maintain Cu homeostasis in the body (DiSilvestro and Cousins 1983).

Other trace minerals such as high dietary Zn, Ca, Cd, Fe, Pb and Ag will affect the amount of copper absorbed by the body. Fischer et al. (1981) found that in rats fed high zinc diets, more copper was bound to a metallothionein-like protein. The authors hypothesized that dietary zinc induces the synthesis of a Cu-thionein in the intestinal mucosa.

In turn, the Cu-thionein which has a higher affinity for copper than zinc, binds copper, thus rendering copper unavailable for transfer to the serum. In contrast, Saylor et al. (1980) showed that dietary supplementation of copper and zinc had no effect on a designated metallothein-like fraction in the cytosol of intestinal cells in sheep. No Cu and little zinc were found in this fraction. Saylor et al. (1980) stated that the lack of response of the metallothionein in the intestinal mucosa to zinc and copper treatments may partially explain the low tolerance for excessive dietary copper by sheep.

An antagonism between Cu, Mo and S exists in the ruminant which also lowers the biological availability of copper (Bull 1980). Sulphur is reduced to sulphide by ruminal microorganisms. Subsequently, sulphide reacts with molybdenum in the rumen to form a thiomolybdate compound, which in the presence of copper creates a Cu-Mo-S complex. Tetrathiomolybdates are not readily degraded in the small intestine and retain a strong affinity for copper (Bremner and Mills 1981). The Cu-Mo-S complex has very low solubility and thus Cu absorption in the lower gut is adversely affected.

## Copper Metabolism - Transport and Storage

Once absorbed from the intestine, copper becomes loosely bound to albumin. The copper-albumin complex is rapidly transported through the portal blood to the liver (Evans 1973). Amino acids such as histidine, threonine and glutamine are also involved in this transportation. Copper is taken up by the hepatic cells by an active transport mechanism (DiSilvestro and Cousins 1983).

H patic copper and zinc binding proteins play a central role in the metabolism of copper (Bremner and Marshall 1974a, b). Metallothionein, a specific low molecular weight protein (MW = 10,000) known for its extremely high affinity for metals (including Cu) is a common form of transportation, storage and detoxification of trace elements in the hepatic and intestinal cells.

Bremner and Marshall (1974a, b) investigated the metallo-proteins of calf livers. With the aid of gel-filtration techniques, these researchers isolated three protein fractions with molecular weights of 75,000, 35,000 and 12,000 respectively. The third fraction was believed to contain metallothioneins because of the similarities to metallothionein found in previous Cd-binding protein experiments. Bremner and Marshall (1974a, b) noted the similarities: relatively low molecular weight (approximately 10,000-12,000), absence of aromatic amino acid residues, high content of sulphuryl groups, high cysteine residue content and high metal content in protein (2%).

Bremner and Marshall (1974a, b) believed that the amount of Cu (and Zn) present in the designated hepatic metallothionein fraction is a function of liver Zn concentration. The absence of such apoproteins in zinc-deficient animals and the relationship between liver Zn and concentration of Cu and Zn in the metallothionein fraction led to the conclusion that zinc was involved in the de novo synthesis of Cu- and Zn-binding proteins (Bremner and Marshall 1974a, b). Zinc has been shown to induce de novo synthesis of metallothioneins in rats injected with zinc preparations (Bremner 1976). Copper, however is very effective in competing with zinc for binding sites since only copper proteins are sometimes

present. In having a greater affinity than zinc for available binding sites on metallothioneins, copper can displace zinc even after zinc has induced the metallothionein's synthesis (Bremner and Marshall 1974a, b).

Saylor et al. (1980) revealed that a very low molecular weight (MW ≈3,500) copper- but not zinc-containing fraction appeared in the supernatant fluid in homogenated liver from sheep fed high copper diets (47 mg/kg DM). Such Cu-proteins are not present in normal or copper deficient sheep. Saylor et al. (1980) theorized that once copper fills available binding sites of the protein fractions, copper becomes loosely bound to smaller proteins and peptides. Limitation of available binding sites on metallothionein and on hepatic proteins may be one explanation for the high susceptibility of sheep to copper toxicosis compared with other species (Saylor et al. 1980).

Ceruloplasmin synthesis and release represents a major pathway of hepatic efflux that is unique to the liver (DiSilvestro and Cousins 1983). Holtzman and Gaumnitz (1970) investigated ceruloplasmin synthesis in rats. They questioned whether the binding of copper to the apoceruloplasmin molecule occurred before or after release of the protein from the liver. Administration of tracer $^{64}Cu$ to copper deficient rats revealed that although $^{64}Cu$ and apoceruloplasmin were both present in the plasma, Cu did not bind to the apoprotein. Holtzman and Gaumnitz (1970) concluded that copper is bound to the protein in the liver before being released into the circulation.

In this experiment, Holtzman and Gaumnitz(1970) also found in copper deficient rats that the release of apoceruloplasmin into the plasma

proceeded at the same rate as copper containing ceruloplasmin in copper normal rats. Concentrations of these apoproteins were only 25% of normal levels because of increased rate of disappearance of apoceruloplasmin. Supplementation of copper did not increase the rate of release of the Cu-protein, but was found to reduce its degradation rate. Holtzman and Gaumnitz (1970) concluded that the presence of copper in newly formed ceruloplasmin increased its stability causing a subsequent rise in ceruloplasmin levels. In contrast, a similar study by Linder et al. (1979) demonstrated that feeding copper to rats increased the rate of ceruloplasmin synthesis without changing the general synthesis of plasma or liver proteins. They hypothesized that failure to observe a stimulation in the rate of ceruloplasmin synthesis by copper in Cu-deficient rats in other experiments, was due to a lag factor. Linder et al. (1979) explained that the effect was not immediate but occurred after a few hours.

In agreement, both groups of researchers found that ceruloplasmin activity was reduced in copper deficient rats (Holtzman and Gaumnitz 1970; Linder et al. 1979). Linder et al. (1979) reported that the Cu-deficient rats had ceruloplasmin with a reduction of 30-50% in enzymatic activity compared to rats in a copper normal state. Eight to sixteen hours after Cu administration, ceruloplasmin activity was restored in the copper deficient rats.

Copper Metabolism - Excretion

Copper incorporation into bile components and subsequent fecal excretion represents another major pathway of hepatic copper efflux in

mammals (including bovine) (DiSilvestro and Cousins 1983). Studies on biliary excretion of copper in the rat have shown that biliary components are classified into two groups (Evans 1973). In the first group, copper is associated with amino acids and small peptides. Amino acids are thought to aid in the membrane transport of copper. In the second group, copper is associated with high molecular weight molecules. Biliary copper bound to the macromolecules are further subdivided by origin, namely: copper that permeates the bile canaliculus and combines non-specifically with proteins in the bile, and protein bound copper that is deposited in the bile as a result of protein catabolism and pinocytosis by hepatic lysosomes (i.e. degraded ceruloplasmin molecules) (Evans 1973).

Endogenous fecal excretion (bile) provides a means of homeostatic control in which the ruminant can adapt to a wide range of dietary intakes of minerals (including copper) and consequently keep levels of the ingested minerals relatively constant within a given tissue (Miller 1974). Urine is not a major pathway for copper excretion in cattle; however, copper binding chelates such as ethylene-diamine-tetracetate (EDTA) can affect urinary output of copper (Miller 1974). In contrast, Hubbs and Oeheme (1982) reported that biliary excretion plays a minor role in homeostasis of copper in sheep. They noted that there is little association between the concentration of hepatic copper and copper content of bile. Furthermore, they suggest that sheep lysosomes are unable to sequester and excrete copper in the bile. Such hepatic characteristics may explain the inability of sheep to maintain Cu homeostasis at relatively low dietary levels of copper (+30 mg/kg).

Previous experiments have shown that a negative relationship exists between the rate of hepatic loss and liver copper concentration in ruminants (Phillippo and Graca 1983). Simpson et al. (1982) hypothesized that low hepatic reserves of copper are conserved by homeostatic regulation of endogenous losses. Therefore, retention of dietary Cu is marginally deficient animals will be higher than in animals with adequate Cu reserves. Phillippo and Graca (1983) determined whether changes in biliary Cu excretion accounted for this relationship.

In a copper depletion experiment on cattle, Phillippo and Graca (1983) fed diets containing 4 mg Cu/kg or 12 mg Cu/kg dry matter to four cattle surgically adapted for collection of bile for approximately 4 months. Bile samples were obtained over an 8 hour period both 4 days before and 3 days after liver biopsy samples were taken. Both bile and liver samples were analyzed for copper. The results indicated that loss of Cu in bile was dependent on hepatic copper content. However, biliary copper excretion accounted for 39.9±2.1% of total copper loss from the liver. Other means of copper loss were not identified.

Miller (1974) stated that endogenous fecal excretion of trace minerals (including copper) by ruminants is the least understood of the many routes of homeostatic control. Experimentally, the separation of unabsorbed element from endogenous sources is difficult. There is little information available concerning the secretions of minerals in bile, pancreatic juice and from the blood into the digestive tract.

Copper Deficiency - General Occurrence

Primary and secondary copper deficiencies in cattle herds have been reported for many regions of the world. In New Zealand, cattle suffer from two common diseases: peat scours and teart disease (Bull 1980). Peat scours or hypocuprosis in ruminants is a result of abnormally low levels of Cu (less than 4 mg/kg DM) and moderately high concentrations of molybdenum (greater than 3 mg/kg DM) in the feed. Similarly, "teart disease" is a result of high concentrations of Mo with normal Cu (about 7 ppm DM) levels. Both mineral imbalances are classified as molybdenosis (Erdman et al. 1978). Animals suffering from molybdenosis exhibit a drain of copper reserves in the body.

Irwin et al. (1979) reported that hypocupremia was responsible for poor feedlot performance of native Hawaiian cattle. Ten poorly performing Herefords originating from the same ranch and all subsequently raised as stockers on lush pastures on the Island of Hawaii were investigated. These cattle failed to gain weight and fatten comparable to their respective pen mates. Trace mineral analysis revealed that serum Cu levels (.07-.12 µg/ml) were well below normal Cu levels (.8-1.5 µg/ml). Clinical signs of hypocuprosis and upper digestive tract lesions were observed in 8 of the 10 animals involved. From forage analysis of 3 pastures on the ranch of origin on the Island of Hawaii, Irwin et al. (1979) showed that these animals grazed forages deficient in copper (2-3 mg/kg) and seasonally high in molybdenum (spring and summer). The molybdenum content of the forage from these 3 pastures ranged from 26-45 mg/kg in the spring to 1-2 mg/kg Mo in the late summer. Forage of

higher molybdenum content originated from soils of a slightly alkaline nature and where annual rainfall exceeds 100 cm.

In the midwest region of the United States, molybdenosis is a potential problem for ruminants grazing land reclaimed from coal mine spoils (Erdman et al. 1978). Firstly, alkaline soil conditions render many minerals including copper unavailable to plants (McDowell et al. 1983). At the same time alkaline soils enhance molybdenum availability to plants (Penumarthy 1978). Secondly, sweet clover (*Melilotus officinalis* and *M. alba*), a common land reclamation plant of coal spoils, is a well known molybdenum accumulator (Erdman et al. 1978). Most plants contain about 1-3 mg/kg molybdenum but Mo accumulator plants in teart pastures contain Mo concentrations as high as 20-100 mg/kg (Penumarthy 1978). Sweet clover, while adaptive to an array of soil and climatic conditions, will not tolerate acidic soils (Gorz and Smith 1973).

Erdman et al. (1978) analyzed copper and molybdenum content of forage samples (predominantly sweet clover) from eight surface mines located in the north central prairie states and southeastern Saskatchewan. The pH of the soil from each mine was also determined. In the survey Erdman et al. (1978) discovered that the copper level in sweet clover samples at all locations were 5.0-9.0 mg/kg DM for forages. Further investigation revealed the molybdenum content in forages from mines containing neutral or slightly acidic spoil soils ranged from 2.6-3.4 mg/kg DM but forages that contained 6.4-13.0 mg/kg DM molybdenum originated from mines having definitely alkaline spoil samples. Erdman et al. (1978) concluded that high concentrations in sweet clover growing on

reclaimed coal mine spoils present a hazard in terms of creating dietary copper imbalance in ruminants grazing predominantly sweet clover pasture.

Primary and secondary copper deficiencies have been recognized in western Canada for the past thirty years. Cunningham (1953) reported that cattle in the Swan River Valley of Manitoba were being adversely affected by high dietary concentrations of molybdenum. Although it is difficult to diagnose a copper deficiency in cattle using visual characteristics, these cattle showed clinical signs of a copper deficiency. The cattle suffered from unthriftiness, emaciation and faded coat color. The molybdenum poisoning was corrected by the daily drenches of two grams of copper sulphate.

More recent studies (1974-1980) were undertaken by the Manitoba Department of Agriculture to investigate the degree and severity of copper deficiency in beef cattle in Northwestern Manitoba (Boila et al. 1984a). An extensive survey was undertaken to measure the trace mineral content, including copper and molybdenum, in forage samples (grass and legumes) from pasture and haylands. About two hundred townships around Duck Mountain Provincial Park and Riding Mountain National Park were included in the survey. This survey revealed that copper concentrations of the forage samples were not sufficient to meet the requirement of cattle. Most legume samples contained about 5 mg/kg DM copper and on average contained more copper than grass forages. Molybdenum and sulphur concentrations were found to be quite variable. Grass and legume samples that contained more than 5 and 10 mg/kg DM Mo, respectively, originated at sites west of a boundary 10 km east of the eastern boundary

of the Ashville Geological Formation, a formation that divides Northwestern Manitoba into two large areas (Boila et al. 1984a). East of this boundary, legume molybdenum content ranged from 5 to 10 ppm. Sulphur values in excess of 0.40 percent were not uncommon.

The extent of a copper deficiency in cattle, based on analysis of copper in blood serum, was also examined (Boila et al. 1984b). Herds of cattle west of the modified Ashville boundary were designated as severely copper deficient. The high concentration of Mo in the pasture forages grazed by these cattle was cited as responsible for the severity of the copper deficiency state (Boila et al. 1984b). One dose of Cu glycinate per head did not prevent a drop in copper serum levels throughout the pasture season. On the other hand, cattle residing east of the modified Ashville boundary were judged as only moderately Cu-deficient. Unlike their western counterparts, these cattle effectively responded to an injection of Cu-glycinate. This treatment prevented a decrease or actually promoted an increase in Cu serum levels of cattle over the summer period on pasture.

Copper Deficiency - Metabolic Aspects

Copper is needed by the ruminant in trace quantities in order to execute several physiological functions. Dietary levels lower than 4 mg/kg DM in the diet will cause a primary copper deficiency in cattle. Secondary copper deficiencies can occur even at adequate copper intakes due to impaired utilization of copper by dietary molybdenum and/or sulphur (Smart et al. 1980). Molybdenosis, hypocuprosis, and conditioned

copper deficiency are all synonymous for a secondary copper deficiency in ruminants (Ward 1978).

Few clinical disorders associated with a trace mineral deficiency are exclusively caused by the respective deficiency. Other factors which are sometimes involved, produce the same specific symptoms (Suttle 1976). However, in a primary or conditioned copper deficient state a beef animal may display certain clinical signs, although such signs may not only be restricted to a copper deficiency. The signs of a copper deficiency in ruminants vary with age, sex, breed and with severity and duration of the deficiency (Underwood 1977).

In the late 1940's, Jamieson and Allcroft (1950) reported that a general unthriftiness in grazing calves was recognized for years by farmers in Scotland and that small amounts of copper sulphate would cure this disorder. Aberdeen-Angus and crossbred West Highland calves were noted to be susceptible to this condition whereas adult cattle of the same breed were not affected. This disorder or unthriftiness was characterized in calves out on pasture for about a month. The calves developed a stilted walk that originated particularly from the back legs. A general graying of coat color took place in black cattle while brown colored breeds turned yellow. Calves affected most severely by the disorder suffered from emaciation, followed by death at 4-6 months. If the calves managed to survive, development remained sub-optimal even after taken off pasture. Jamieson and Allcroft (1950) demonstrated that such symptoms were due to a copper deficiency in the cattle. Nevertheless, the cattle were thought not to be affected by a primary copper deficiency, but rather to a molybdenum antagonism which interfered with

copper metabolism.

Smart et al. (1980) reported similar findings in four seven month old Simmental calves which represented calves from 200 cows on pasture. The animals were unthrifty and had a dull, dry hair coat. They walked with stiffness and suffered from lameness. Smart et al. (1980) observed the legs of these calves were straight with abnormally enlarged distal metacarpal and metatarsal joints. These calves suffered from both gastrointestinal and pulmonary parasitism. Analysis of the blood revealed all calves had a mild microcytic normochromic anemia. Plasma copper concentrations were low (0.50 μg/ml) and calves were deficient with respect to hepatic copper levels (2.12 mg/kg, wet basis).

As cited in these two examples, abnormal bone development is common in the growing ruminant in a copper deficient state. Similarly, Smith et al. (1975) observed that half of the calves (5 to 12 month old) from herds grazing high molybdenum soils suffered to some degree of lameness. All calves had a visible swelling in the metacarpal and metatarsal areas of the legs. Adult cattle showed no signs of lameness. Radiographic examination of a longitudinal section of the metacarpus and metatarsus from selected calves revealed that an abnormal widening of the cartilaginous growth plate (epiphyseal plate). Irregular appearance in some areas on both the metaphyseal and epiphyseal edge (of the epiphyseal plate) and a lipping of the bone at the metaphysis was noted. Likewise, Smart et al. (1980) reported a similar abnormality in copper deficient Simmental calves, such that the physis of the metacarpal and metatarsal bones was irregular due to an extension of the cartilage into

the metaphysis. Smith et al. (1975) also noted cortical thickness and the trabecular pattern of the diaphysis were normal. The cortical thickness and trabecular pattern of the epiphysis and the density and width of the subchrondral bone were also normal.

Suttle et al. (1972) examined the proximal and distal extremities of the tibia and the third and fourth costochondral junctions of 10 week old lambs. These lambs were reared on either Cu-depleted or Cu supplemented suckled ewes. The majority of the unsupplemented lambs and only one supplemented lamb showed osteoporotic lesions. The tissue copper content in the unsupplemented lambs was well below corresponding tissue values in the lambs from supplemented ewes (152 vs. 330 mg/kg, DM for liver; 9.6 vs. 16.9 mg/kg, DM for brain). Histological examination of the lesions from the affected lambs revealed that bone trabeculae in the central part of the metaphysis were reduced in number and were absent in the most severe cases. Osteoblastic activity and deposition of bone matrix were significantly reduced or had ceased. The structural integrity of the mature trabecule was extremely delicate. Osteoporosis in the unsupplemented lambs was most severe in the metaphyseal region. Unlike Smith et al. (1975), Suttle et al. (1972) found no thickening or overlapping of the epiphyseal cartilaginous plate in the lambs.

Osteoporotic lesions in the bones of Cu-deficient ruminants is a result of reduced activity of monoamine or lysyl oxidase (Mills et al. 1976). Monoamine oxidase, a copper dependent enzyme, catalyzes the oxidative deamination of lysine residues. Oxidative deamination of lysine results in crosslinkage of polypeptide chains. Such crosslinkage is important in the stability of collagen, the structural protein of

bone, connective tissue and elastin, a major protein of vascular tissue (Evans 1973).

Similar to bone disorders, vascular fragility has been associated with copper deficiencies in ruminants. Mills et al. (1976) fed five Friesian cattle a semi-synthetic diet containing less than 1 mg/kg, DM Cu. The animals were copper deficient with respect to blood and liver copper contents after approximately 3 months on the diet and a decrease was noted in monoamine oxidase activity in the plasma. One steer died because of rupture of the posterior vena cava. Mills et al. (1976) also found several structural defects in the cardiovascular wall tissue of all five steers, decreased content of interlaminar collagen, and in the aorta, degeneration of the smooth muscle. Suttle and Angus (1976) discovered minor abnormalities in the thoracic aortas of three Cu-depleted newborn calves. In one aorta, the elastin fibers were deficient, swollen and fragmented, and the wall of another aorta of a Cu-deficient calf was thinner than normal.

Anemia is another common effect of prolonged copper deficiency in ruminants. Mills et al. (1976) observed a small decline in hemoglobin concentration in cattle fed a copper deficient ration for 262 days. No significant differences in erythrocyte count were recorded. Higher concentrations of plasma iron were maintained in the copper supplemented animals compared to the copper depleted animals. Suttle and Angus (1976) also reported a tendency for hemoglobin concentration to be lower in Cu-depleted calves after 18 weeks on a copper deficient diet. On the other hand, Smart et al. (1980) recorded mean corpuscular hemoglobin

concentrations in Cu-deficient calves as normal (normochromic) but depressed packed cell volume in the plasma.

In rats, iron absorption by the intestinal mucosa was unaffected by copper deficiency, but the transfer of iron to plasma was impeded (Sourkes et al. 1968). Throughout the absorption process in the intestinal mucosa, ionic iron is mainly in the ferrous form ($Fe^{+2}$). Once in the plasma, $Fe^{+2}$ is rapidly oxidized to $Fe^{+3}$ by the copper dependant enzyme, ceruloplasmin (Osaki et al. 1966). The ferric iron is then attached to a specific binding protein, transferrin, in the plasma and is transported to the bone marrow. Transferrin exclusively supplies iron to the bone marrow which is the site of hemoglobin synthesis (Osaki et al. 1966). Tephly et al. (1978) point out that aside from ceruloplasmin, copper also plays a more direct role in the synthesis of hemoglobin. Copper has been shown to stimulate the activity of ferrochelatase, an essential enzyme catalyzing reactions in the heme synthetic pathway.

An animal deficient in copper, and consequently ceruloplasmin, supposedly will not be able to synthesize iron transferrin fast enough to supply the iron required for the formation of hemoglobin and other iron containing proteins (Friedan 1981). Mills et al. (1976) demonstrated a decrease of both hemoglobin and ceruloplasmin (ferroxidase) levels in primary copper deficient cattle. Bingley and Anderson (1972) revealed molybdenum in the pastures (2-9 ppm, dry weight) readily reduced ceruloplasmin activity and caused a macrocytic hypochromic (low Hb) anemic condition in hypocuprotic calves. However, Bingley (1974) observed plasma ceruloplasmin levels remained unchanged while hemoglobin levels

dropped 30% in secondary copper deficient or Mo + $SO_4$ fed sheep. Copper in the erythrocytes was depressed to 10% of the normal concentration while plasma total Cu was significantly elevated.

In the 1930's, it was found that a nerve disorder reported in sheep and cattle in Florida, Holland and Australia was due to a copper deficiency, and could be prevented by dietary copper supplementation (Bull 1980). This disease is known as slat sick, lechsucht or enzootic ataxia. In lambs and sometimes in cattle, a condition known as swayback or neonatal ataxia has been reported (Hansard 1983). Neonatal ataxia commonly affects lambs born to copper deficient ewes either at the time of birth or delayed for several weeks after birth (Underwood 1977). Symptoms of the disorder include uncoordinated movements of the hind limbs, a staggering gait and swaying of hind quarters as the disease develops.

Neonatal ataxia in ruminants is a result of demyelination of the motor neurons (Ward 1978). In a normal motor neuron, a myelin sheath wraps the nerve axon such that it lies between most of the Schwann-cell cytoplasm and the motor nerve axon (Keeton 1972). The Schwann-cells play a role in nutrition of the nerve fibers while the myelin sheath functions in speeding up the conduction of nerve impulses in the axon. Many researchers who have studied demyelination, believe that ataxia in the neonate from a copper deficient dam is due to myelin aplasia rather than myelin degeneration (Bull 1980). During late gestation maternal copper deficiency depresses activity of fetal cytochrome oxidase, a Cu-dependent enzyme, leading to inhibition of aerobic metabolism and phosphosynthesis (Hansard 1983). Since myelin is composed largely of

phospholipids, myelin production ceases. Consequently, fetal nerve maturation is incomplete.

Achromotrichia or lack of pigmentation in hair has been widely reported in copper deficiency studies of ruminants. Mills et al. (1976) observed black cattle fed a diet deficient in copper developed a grey-brown cast over normally black areas of the coat (165 days). Later on in the experiment (225 days), a 'spectacle eye' pattern of depigmentation was seen. Straw-colored hair coat or 'yellow calf' is also commonly seen in secondary copper deficient cattle grazing high molybdenum pastures (Irwin et al. 1979). Suttle and Angus (1976) reported some depigmentation in copper deficient Jersey calves. However, they demonstrated that loss of hair pigment may not be a satisfactory criteria for detecting copper deficiency in cattle because of natural variations in the pigmentation of the hair coat of Jersey calves.

Tyrosinase, a Cu-containing polyphenyloxidase, catalyzes two reactions in the conversion of tyrosine to the pigment, melanin (Evans 1973). A genetic absence of tyrosinase has been shown to contribute to albinism, and to achromotrichia.

Low fertility, because of delayed or depressed estrus, has been shown to occur in cows grazing copper deficient pastures (Underwood 1977). Bertelsen (1982) reports that researchers in Texas find that reduced conception rates and embryonic and fetal mortality are the most common reproductive syndromes of copper deficiency in cattle. Likewise, Campbell et al. (1976) reported that testicular growth and development was retarded in bull calves fed a high molybdenum/low Cu diet.

Improvement in testicular maturity occurred when such bulls were given a high copper ration. Copper is important for normal reproductive function but the mode of action is unclear.

Copper and Molybdenum - Mo in the Ruminant

The natural presence and function of molybdenum in the body is greatly overshadowed by molybdenum's antagonistic effect on the metabolism of dietary components. Aside from its antagonistic relationship with copper, molybdenum is an essential trace mineral for plants and animals. However, the adverse effects of a molybdenum deficiency upon ruminants have never been proven (Penumarthy 1978). The biochemical function of molybdenum centers around three enzymes: xanthine, aldehyde and sulphate oxidase (Hansard 1983). Xanthine oxidase which contains 8 atoms of Mo and 8 of Fe per molecule, and aldehyde oxidase are involved in the electron transport chain (sp. cytochrome C) in the cell. Xanthine oxidase functions in purine metabolism and also in the release of Fe into the plasma from hepatic ferritin through riboflavin derivatives (Hansard 1983).

The highest concentrations of molybdenum are found in the liver, kidney, bone, muscle and skin (Hansard 1983). Liver and kidney contain more molybdenum than other organs but unlike copper, molybdenum does not accumulate in the liver (Penumarthy 1978). On a wet matter basis, the liver and kidney normally contain 0.74 and 0.27 ppm Mo respectively. Cattle grazing pastures adequate in copper and deficient or low in molybdenum, have a normal Mo blood level of 6 µg/100 ml (Underwood 1976).

Until a maximum value if obtained, blood Mo levels are strongly correlated to the level of daily ingested molybdenum. In the cow, the molybdenum content of the milk is bound entirely to the Mo containing enzyme, xanthine oxidase (Penumarthy 1978). Enzyme assays reveal xanthine oxidase activity is proportional to the milk's molybdenum content.

A definite molybdenum requirement for most species has not been established. Early experiments (1950's) performed on rats and chicks, fed semi-purified diets, have shown Mo requirements to be below 0.2 ppm (Underwood 1976). Lambs fed a semi-purified diet containing 0.36 ppm Mo showed a growth response when the ration was supplemented with 2 ppm Mo. Field studies indicate that sheep grazing pastures containing less than .36 ppm Mo, (DM), accumulate copper which may lead to chronic copper toxicity (Underwood 1976).

Although much attention has been drawn to the feeding of dietary molybdenum to the bovine, a definitive dietary level of molybdenum that results in a molybdenum toxicosis has not been established. Ward (1978) reported that on fresh pasture, the minimum toxic concentration for cattle is 20 mg/kg as fed and that 100 mg/kg as fed of Mo will cause signs of molybdenum toxicosis. Penumarthy (1978) pointed out that 60 mg/kg soluble molybdates/head/day will produce molybdenum toxicity in cattle. Vanderveen and Keener (1964) acknowledged that as little as 2 mg/kg, DM of Mo, when copper levels were 3.8 to 4.2 mg/kg, DM in forage has been shown to cause molybdenum toxicity in cattle. They added that in general, toxic levels for cattle range between 6 to 36 mg/kg, DM. Lactating dairy cows were fed a diet containing 6 mg/kg, DM of copper supplemented with sodium molybdate by Huber et al. (1971). Signs of molybdenum toxicity

were observed in the cows consuming 173 to 200 mg/kg DM molybdenum, whereas none of the cows receiving 53 to 100 mg/kg DM experienced any adverse effects.

Cattle have the least tolerance to high dietary molybdenum compared with other farm species. Dairy cattle and young stock apparently are more susceptible to molybdenum toxicity than beef cattle (Penumarthy 1978). Horses fail to show any clinical signs of molybdenum toxicity on "teart pastures" (40-50 ppm Mo) whereas cattle are severely affected. Hogs have ingested diets with 1000 ppm of molybdenum without any adverse effects whereas 10-20% of this level will cause severe scouring in cattle (Underwood 1976).

Diarrhea is the most common characteristic affecting an animal with molybdenum toxicosis (Penumarthy 1978). Other signs of Mo toxicity include anorexia, loss of condition, followed by loss of hair color, anemia, stiffness and bone brittleness (Ward 1978). Changes in coat color and anemia are possible manifestations of a secondary copper deficiency whereas molybdenum interference with phosphorus metabolism leads to lameness, joint abnormalities, and osteoporosis (Underwood 1976). High molybdenum intakes by cattle can also lead to death. Cook et al. (1966) reported that three 264 kg steers died after receiving 3.0 mg of molybdenum per kg of body weight daily for 100 days.

## Copper and Molybdenum - Antagonistic Interactions

Many experiments have been recorded on the effects of molybdenum on copper status in cattle, either on pasture or in drylot. The results

of these studies indicate a variety of responses.

Bingley and Anderson (1972) studied the effects of high molybdenum content of forages grazed by beef calves. They allotted several beef calves to graze two pasture areas, designated as paddock A and paddock B. The average concentration of copper and molybdenum in the pasture forages were respectively 3.96 ppm and 6.70 ppm in paddock A and 4.71 ppm and 4.52 ppm in paddock B. The pasture inorganic sulphate level was 0.21% and 0.26% respectively. All cattle remained on paddock A or B for sixteen weeks before being chosen for slaughter or moved to paddock C, a pasture area where the forages contain relatively lower concentration of molybdenum (1.62 ppm) and a higher copper (7.36 ppm) content. Some of the calves in paddock A and B were also injected with a copper glycinate preparation during an initial phase of the study. Initial blood determinations for the Cu glycinate treatment and blood samples of untreated calves obtained a month before slaughter revealed that total blood copper values between calves from paddocks A and B were not singificantly different. However, when differences did arise, the blood Cu was inversely related to the molybdenum content of the pastures. Data from slaughtered untreated calves from both pastures after sixteen weeks showed blood copper levels of 0.44±0.13 µg/ml and a hepatic copper content of less than 12 ppm, dry matter basis, (<3.0 ppm, wet wt.). Such animals were classified as copper deficient based on the criteria by Puls (1981).

Similarly, Huber et al. (1971) supplemented sodium molybdate to a basal diet (6 ppm Cu) to establish various molybdenum treatments; 0, 53 and 173 ppm Mo for lactating dairy cows. The results demonstrated

that groups receiving the molybdenum supplemented diets had a small transient rise in hepatic copper levels for about one month. Afterwards, a rapid decline followed until the end of the six month experiment. Cows on both Mo treatments exhibited an increase in milk Cu as well as a rise in kidney Cu levels. In contrast to Bingley and Anderson (1972), these authors reported blood copper was not altered by the Mo treatments. Subsequently, liver, blood and milk molybdenum levels significantly rose and were somewhat proportional to molybdenum intakes. Experimental cows on the highest molybdenum level (173 mg/kg DM) had overt molybdenum toxicity (i.e. diarrhea) whereas animals consuming the 53 mg/kg DM molybdenum and control diets exhibited no such disorder.

Vanderveen and Keener (1964) reported that dairy heifers consuming basal diets (<2 ppm mg/kg DM) containing 5 to 50 mg/kg DM molybdenum, and no added sulphate sulphur, did not develop molybdenum toxicosis. Heifers on the lower molybdenum (5 to 20 mg/kg DM) diets with 0.3% added sulphate sulphur did not develop the clinical signs associated with a molybdenum toxicity. However, heifers on a 50 mg/kg DM molybdenum and similar sulphate level suffered from alopecia (loss of hair) and achromatrichia (loss of hair pigment). The molybdenum supplemented animals had significantly depressed liver copper levels whereas animals consuming diets without added molybdenum showed a rise in hepatic copper (regardless of sulphur intake). Serum copper levels dropped off slightly for all animals on Mo treatments.

Clawson et al. (1972) studied the effects of copper sulphate and injectable copper glycinate and their interrelationship with dietary

molybdenum as affecting copper status of beef heifers. Six treatments were established and assigned to the heifers for about four months. The treatments included: a basal diet (containing 2.5 mg/kg DM Mo and 4.5 mg/kg DM Cu respectively), the basal diet in single combination with 1 g of dietary copper sulphate or with a subcutaneous injection of 2 ml copper glycinate (120 mg copper) or the basal diet supplemented with molybdenum (100 mg/kg DM) only, or in combination with either dietary copper sulphate or injectable copper glycinate.

In the experiment, Clawson et al. (1972) found that plasma copper levels were initially elevated by both copper sources and/or the dietary molybdenum supplement. However, after plateauing, the plasma copper levels dropped to near control levels toward the end of the experiment in heifers receiving either a copper treatment or a molybdenum supplement. Copper concentration of plasma in animals receiving a combination of molybdenum and copper (regardless of source) declined only slightly. Both copper sources initially increased liver copper levels regardless of dietary molybdenum. By the end of the experiment, the Cu glycerate injected animals (with or without Mo) exhibited a decline in hepatic copper to control levels. Although hepatic copper continued to rise in the copper sulphate animals, molybdenum was shown to limit this elevation. Animals on the basal diet supplemented only with molybdenum had an initial rise in liver Cu but by the end of the trial such copper values were comparable to control hepatic levels.

Copper and Molybdenum - Cu:Mo Ratio

Since the requirement of copper for cattle is uncertain because of varying amounts of dietary molybdenum and/or sulphate in the diet, many researchers have used the dietary ratio of Cu to Mo to assess and meet the animal's copper requirement. Miltimore and Mason (1971) suggested that a dietary Cu/Mo ratio above 2:1 will meet the copper requirement of cattle but that lower ratios are associated with a secondary copper deficiency. Ward (1978) listed a Cu/Mo ratio of 2:1 or less as one of four classes of feed which will produce a conditioned copper deficiency. Furthermore, Miltimore and Mason (1971) stated that the absolute Cu and Mo concentrations are unimportant in the Cu/Mo ratio, although high molybdenum concentrations associated with low Cu concentrations produce undesirable Cu/Mo ratios of less than 2:1.

Miltimore and Mason (1971) studied the copper and molybdenum concentrations and Cu/Mo ratios in ruminant feeds throughout British Columbia. Almost all the samples contained 10 mg/kg DM or less of Cu, and about one-seventh of the samples were below 3.0 mg/kg DM Cu. Legume feeds had a mean Cu content of 7.5 mg/kg DM, more than twice the Cu content of sedge hay (3.3 mg/kg DM). Most of the feed samples (85%) contained less than 3.0 mg/kg DM Mo and about one-third contained less than 1.0 mg/kg DM Mo. Miltimore and Mason (1971) noted that as Cu concentrations increased in all feeds the Cu/Mo ratio also increased. For individual samples, Cu/Mo ratios ranging from 0.1 to 52.7 were found. About one-fifth of all samples had Cu/Mo ratios of 1.9 or less (under the critical 2:1 level). Sedge hays had a mean Cu/Mo ratio of 2.1,

whereas grass hays had a mean Cu/Mo ratio of 4.4. Grains, legume feeds, corn silage and oat forage had mean ratios of 7.4, 6.7, 6.3 and 5.0 respectively. From these data, the low concentrations of copper in the feed point out that cattle in British Columbia are subject to a simple copper deficiency rather than molybdenosis.

The critical Cu/Mo ratio of 2:1 is supported by many examples in the literature. Although specific Cu/Mo ratios are rarely reported, individual Cu and Mo concentrations in cattle diets are frequently noted (Miltimore and Mason 1971). Miltimore et al. (1964) observed that cattle grazing pastures with a Cu/Mo ratio of 4.3 did not exhibit any clinical signs (i.e. diarrhea) associated with a secondary copper deficiency. However, cattle grazing a pasture with a Cu/Mo ratio of 2.3 showed some scouring while cattle on a pasture with a Cu/Mo of 1.0 suffered from severe molybdenosis. Thornton et al. (1972a, b) found that cattle grazing two pastures with a mean Cu/Mo ratio of 1.06 and 1.82 respectively (calculated from herbage Cu and Mo content) had blood copper values which were inversely proportional to herbage molybdenum content. Bingley and Anderson (1972) reported deficiency concentrations of blood and liver copper in beef calves grazing pastures which contained herbage with Cu/Mo ratios of less than 2.0. Such pasture samples contained 2.4-6.7 mg/kg DM Cu and 2.1-9.2 mg/kg DM Mo. Ward (1978) pointed out that subclinical copper deficiencies in cattle are common in several areas of the British Isles and Netherlands. In such areas, the Cu/Mo ratios fall well below 2.0 (1:1 to 1:1.7).

Givens and Hopkins (1981) recorded decreased whole blood copper content in cattle grazing pastures with Cu/Mo ratios above 2.0 (2.8-3.8).

Forage samples from these pastures had Cu and Mo concentrations of approximately 10.0 and 3.0 ppm respectively. Campbell et al. (1976) suggested that a 5:1 Cu:Mo ratio in the diet is more appropriate to balance the effects of molybdenum on copper levels in cattle.

The major drawback in using Cu/Mo ratios to assess the copper availability of feed is the failure to acknowledge other copper related interactions, especially with sulphate (Suttle 1980). Although the Cu/Mo ratios in the pasture reported by Thornton et al. (1972a, b) was an undesirable 1:1, there was sufficient sulphate in the herbage to potentiate the adverse effects of molybdenosis upon copper status in cattle. Likewise in the pastures with Cu/Mo ratios above 2.0 as noted by Givens and Hopkins (1981), the sulphur level in many of the pastures was very high (0.3-0.4%).

## Copper and Molybdenum - Mechanism of Interaction

A possible mechanism which illustrates the antagonistic relationship between copper and molybdenum in ruminants has been extensively investigated by Dowdy and Matrone (1968a, b). They proposed that the antagonism between copper and molybdenum is due to the formation of a copper-molybdenum complex. Dowdy and Matrone (1968a) characterized the nature of the Cu-Mo complex by synthesizing the compound in vitro. An insoluble precipitate was formed when solutions of sodium molybdate and copper sulphate were mixed at neutral pH. The concentration of the newly formed Cu-Mo complex in solution (as measured by absorbance at 340 mμ) and the weight of precipitate indicated a molar ratio of Cu to Mo of 4:3. The

presence of sulphate in the precipitate was not detected. Later experiments by Dowdy and co-workers revealed this complex to be similar to the mineral lindgrenite; 2 $CuMoO_4 \cdot Cu(OH)_2$, commonly known as cupric-molybdate (Huisingh and Matrone 1976).

Dowdy and Matrone (1968b) studied the existence of a Cu-Mo complex in vivo by injecting a sheep with a Cu-Mo complex synthesized from $^{64}Cu$ and $^{99}Mo$. The results of the study showed that rates of removal from the blood of Cu and Mo (originating from the complex) were equal. The removal rate was more rapid in this animal than the removal rate of molybdenum in another sheep injected with $^{99}Mo$ alone. The Cu-Mo complex was also excreted via urine more slowly than molybdenum alone. Similarly, Clawson et al. (1972) found that plasma copper was higher when molybdenum was included in the diet of beef calves. Since molybdenum supplementation did not increase hepatic copper to the same degree as plasma copper, these researchers suggested that a Cu-Mo complex exists in the calves blood. Clawson et al. (1972) also proposed that Cu-Mo complexes were formed in the digestive tract and that copper absorption was limited because the total blood copper in comparison to dietary copper was small. Dowdy and Matrone (1968b) found in pigs that Cu in the Cu-Mo complex appeared to be absorbed and transported but was unavailable for ceruloplasmin synthesis.

Suttle (1974a) theorized that elevated plasma copper as a result of molybdenum feeding may be in the form of a Cu-Mo complex, originating in the gut tissues. This complex may be excreted by the kidney as urinary output of Cu rises in sheep and cattle fed molybdenum. Bremner

and Young (1978) also found that Cu and Mo tend to accumulate together in the same renal sub-fractions after subcellular fractionation and gel filtration of the cytosol. They proposed that Mo-induced changes in plasma and tissue (kidney) distribution is a consequence of the formation of molybdenoproteins with a high affinity for Cu. Huber et al. (1971) found that high levels of dietary molybdenum tended to elevate kidney Cu while liver Cu declined.

Dowdy and Matrone (1968a) also demonstrated that molybdenum additions to incubation solutions reduced the in vitro uptake of radioactive tracer $^{64}Cu$ by rat liver and kidney slices. It was suggested that the molybdate may have interfered with the absorption process of copper by the liver slices or that molybdate may have complexed with copper before absorption.

Cu-Mo-S Interactions - Sulphur Metabolism in the Ruminant

Inorganic sulphate is a necessary component for the de novo-synthesis of sulphur amino acids and plays a significant role in molybdenum and copper metabolism in the ruminant (Gawthorne and Nader 1976). In the rumen, inorganic sulphur is metabolically reduced to sulphide by microorganisms in the digesta. This reduction process of sulphate follows two main pathways, namely the assimilatory and the dissimilatory reductive pathways (Goodrich 1978).

Into the assimilatory pathway, inorganic sulphate is initially converted into an activated S compound, adenylsulfate, by a series of three enzymatic steps (Stanier et al. 1976). Such reactions require the

expenditure of three high energy phosphate bonds. In the final reaction the reduction of sulphite intermediate to sulphide is catalyzed by a complex flavometallo-protein sulfite reductase. Many types of micro-organisms are able to reduce sulphate via assimilation, although this type of sulphate reduction represents only a small proportion of total cell production (Goodrich 1978). Sulphide produced via assimilation is directly incorporated in sulphur containing organic compounds of which cysteine is the major end product.

Conversely, dissimilatory reduction of sulphate occurs on a large scale in microbes which possess the pathway (Goodrich 1978). Anaerobic microbes from the genus Desulfotomaculum (sporeformers) and from the genus Desulfovibrio (nonsporeforming, strict anaerobes) are the two main microbial populations in the rumen which reduce sulphate to sulphide via the dissimilatory pathway (Stanier et al. 1976). The microbes utilize the sulfate in anaerobic respiration as the terminal electron acceptor (Peck et al. 1959). As a result of sulfate conversion to sulfide, four high energy bonds are produced.

In the first step of the dissimilatory process, adenosine-5′-phosphosulfate (APS) and pyrophosphate (PP) are formed from ATP and sulfate (Stanier et al. 1976). This step is catalyzed by the enzyme, ATP sulfurylase. ATP-sulfurylase activity is inhibited by Group VI anions; $MoO_4^{2-}$, $SeO_4^{2-}$, $WO_4^{2-}$ and $CrO_4^{2-}$ (Peck et al. 1959). APS plus 2e (donated from electron carrier: cytochrome $C_3$) is then converted into AMP + $SO_3^{2-}$ (sulfite) (Stanier et al. 1976). Finally, sulfite is reduced to sulfide by the microbes in a three step process (electron also donated

by cytochrome $C_3$), accompanied by the recycling of sulfite. These three reduction steps are catalyzed by a green protein, desulfoviridin (Stanier et al. 1976). This sulfite reductase is abundantly found in the Desulfovibrio organisms but not in Desulfotomaculum microbes. However, the Desulfotomaculum utilize a similar sulfite reductase for the conversion of sulfite to sulfide.

Sulfide produced via the dissimilatory pathway is assumed to be released by the microorganisms into the ruminal sulfide pool (Goodrich 1978). Sulfide in the rumen is rapidly absorbed through the ruminal wall, oxidized to sulfate in the tissues for metabolic purposes, and any excess is excreted via the urine (Huisingh et al. 1973).

Cu-Mo-S Interactions - General Antagonism

Interactions among copper, molybdenum and sulphur in ruminants are widely documented in the literature. Nevertheless concrete evidence concerning the presence and mechanisms of such interrelationships are presently unclear (Goodrich 1978). For the last 30-40 years high dietary molybdenum has been shown to adversely affect ruminants in a marginal or copper deficient state. Whether inorganic sulfate can induce a copper deficiency under similar conditions or potentiate molybdenosis in ruminants is highly controversial. Cattle are noted to be more sensitive to high dietary molybdenum then sheep (Goodrich 1978). Many studies have reported that molybdenum will create a secondary copper deficiency in cattle regardless of sulphate level. On the other hand, experiments performed on sheep tend to show that molybdenosis alone cannot cause a secondary copper deficiency without added inorganic sulphate (Bremner

and Young 1974; Suttle 1975).

Cook et al. (1966) studied the effects of sodium sulphate, fed in combination with inorganic molybdenum, on plasma copper levels of beef heifers on pasture. Half of the group of experimental heifers received a daily molybdenum supplement of 3.0 mg/kg body weight, whereas the others received the same Mo supplement plus 34 gm of sodium sulphate/ day for approximately two weeks. The results showed that sulphate significantly lowered plasma molybdenum, but had no significant effect on plasma copper levels. Likewise, Huber et al. (1971) fed a basal diet that contained 6 mg/kg DM of Cu and was supplemented with sodium molybdate from 53 to 300 mg/kg DM molybdenum. When sulphate was added to the diet, liver and blood molybdenum concentrations were depressed but sulphate had no consistent effect on liver copper levels.

Givens and Hopkins (1981) observed that cattle grazing forages of adequate copper content (10 mg/kg DM), molybdenum concentrations of 3.0-4.0 mg/kg DM and of broad sulphur content (0.07-0.41%) had depressed blood Cu levels of 0.02-0.03 mg/ml by the end of the pasture season. Based upon the Cu, Mo and S concentration of such pasture forages, these researchers calculated the availability of dietary Cu to cattle on pasture. Givens and Hopkins (1981) used the equation as follows:

$$\left[\log \text{TA Cu} = -0.0019\,\text{Mo} - 0.755\text{S} - 0.0131(\text{MoxS}) - 1.153\right] \times \text{Total conc. of Cu in the forage} = \text{Total available Cu}$$

where TA Cu = true availability of Cu and Mo and S are dietary concentrations as mg/kg DM and g/kg DM respectively. Hopkins and Givens

(1981) concluded from this equation that the availability of dietary copper in the pastures under study was inadequate to maintain copper plasma levels in cattle.

Cu-Mo-S Interactions - Cu and S

Sulphur compounds either from sulphate or S containing amino acids are broken down and reduced to hydrogen sulphide by microorganisms in the rumen. This sulphide may continue with dietary copper to yield copper sulphide, which is not absorbed by the ruminant gut (Huisingh et al. 1973). Previous studies have suggested that copper utilization is restricted by sulphide, by depressing copper solubility in the digestive tract through the precipitation of insoluble copper sulphide (Goodrich 1978). The concentration of soluble copper in the rumen is inversely proprotional to ruminal sulphide levels. It also has been theorized that copper sulphide could be formed in the blood or in the tissues (Huisingh et al. 1973).

Suttle (1974b) studied the effects of dietary sulphur on the availability of dietary copper in copper-repleted, hypocupraemic ewes. In one trial, he fed three groups of ewes a basal diet supplemented with 4 mg/kg diet Cu (as $CuSO_4$). One group received no sulphur addition while the second and third groups received 2.5 g of S/kg as sodium sulphate or methionine for about one month. At the end of the experiment, the sheep receiving the S-supplemented diets exhibited similar results. Organic and inorganic sulphur reduced the response of plasma Cu due to copper repletion ($CuSO_4$) by 56%. The rumen sulphide concentration was elevated both in methionine (12.8 mg/ℓ) and sodium sulphate (11.2 mg/ℓ) treated

ewes compared to untreated-S animals (2.3 mg/ℓ).

With further experimentation, Suttle (1974b) demonstrated that dietary sulphur failed to inhibit the positive response of plasma Cu in hypocupraemic ewes being repleted by a continuous intravenous infusion of Cu (thus bypassing the gut). In another trial, Suttle (1974b) compared CuS and $CuSO_4$ as copper supplements for hypocupraemic ewes. While plasma Cu rose in $CuSO_4$ treated ewes, CuS animals showed no change in plasma Cu. From these experiments, Suttle (1974b) concluded that the utilization of Cu is impaired by sulphur as sulphide due to the formation of insoluble CuS in the gut. He suggested that conditions in the rumen as well as in the omasum and abomasum favored CuS formation.

Ward (1978) acknowledged that copper deficiency symptoms in ruminants grazing pastures apparently adequate in copper are caused by the interaction between Cu and S. He stated that these cases occur only in grazing situations and that if the forage is processed into hay, the problem disappears. Ward (1978) reported that protein (25-30% DM) in the lush pasture is very soluble and readily hydrolyzes in the rumen. As a result, sulfide (from S-amino acids) is produced which combines to produce insoluble CuS. As a dried hay rather than lush pasture, protein content and solubility is lower, therefore less ruminal sulphide is produced from green forage.

Nikolic et al. (1983) examined the influence of different concentrations of molybdenum and copper on sulphide concentrations in bovine rumen contents, incubated in vitro. By adding copper acetate solution, these researchers elevated the copper concentration of the incubation

mixture, from 0.4 to 1.8 mg/ℓ. As a result, the sulphide concentration was significantly reduced (1.55 mg to 1.35 mg/ℓ, after two hours of incubation) when copper (1.8 mg/ℓ) was added to the medium. However such a Cu addition did not affect the utilization of sulphide for microbial protein synthesis. Nikolic et al. (1983) summarized that the drop in sulphide concentration was most likely due to the precipitation of insoluble copper sulphide.

Cu-Mo-S Interaction - Mo and S

Molybdenum is a group VIB element. Although it can exist in several oxidation states, it preferably binds to four oxygens and exists as the oxy-anion, molybdate ($MoO_4^{2-}$) (Huisingh and Matrone 1976). Sulphate and molybdate are both tetrahedral anions with the same charge. Such chemical similarities suggest an antagonism could exist between dietary molybdenum and sulphur in the ruminant (Huisingh et al. 1973). Depending on the nature of the Mo-S interaction, the sulphur level in the diet and the copper status of the animal, molybdenum can either intensify or alleviate copper deficiency symptoms observed in ruminants (Goodrich 1978).

Huisingh et al. (1976) recalled an earlier in vitro study in which molybdenum and radioactive tracer, $^{35}SO_4^{2-}$ were added to washed cell suspensions of rumen microorganisms of sheep, fed a non-protein, urea diet containing sulphate. After incubating the mixture, the researchers found that molybdate concentrations as low as 5 ppm could cause a 50% inhibition of the sulphate reduction; specifically $^{35}SO_4^{2-}$ conversion to $H_2{}^{35}S$.

Molybdate inhibition of sulphate reduction is believed to be due to the competition between molybdenum and sulphate at the first stage of sulphate activation (Gawthorne and Nader 1976). Under normal circumstances, ATP sulfurylase catalyzes the activation of sulphate by ATP to form adenosine-5′-phosphosulphate. This enzyme has been shown to be irreversibly inhibited by molybdate in rumen microorganisms (Huisingh et al. 1976). Wilson and Bandurski (1958) demonstrated that molybdate irreversibly cleaves ATP to form adenosine-5′-phosphomolybdate (APMo). APMo is more unstable than APS (yield from sulphate) and thus APMo hydrolyzes to form AMP and molybdate. Such molybdate inhibition studies were mainly performed under in vitro conditions.

Huisingh et al. (1975) fed two separate groups of fistulated ewes on purified diets containing either sodium sulphate or methionine. After a pre-experimental period, selected animals were fed a 50 mg/kg Mo supplement. Ruminal samples were obtained and assayed for sulphide production. Ruminal samples obtained from the sheep before dietary Mo addition, were also incubated in vitro with methionine or sulphate as a substrate in the presence or absence of 4.5 mg/kg Mo as $Na_2MoO_4 \cdot 2H_2O$. Dietary molybdenum was shown in vivo to inhibit the production of sulphide from sulphate, but to elevate sulphide levels in sheep on the methionine treatment. These researchers believed that molybdenum decreased sulphate reduction by inhibiting the first enzyme in the dissimilatory sulfate-reducing pathway, ATP-sulfurylase. Stimulation of $H_2S$ production in methionine fed sheep by the addition of dietary molybdenum, was attributed to stimulation of the number of $H_2S$ producing microorganisms.

When pre-experimental rumen samples from sheep fed either sulphate or methionine were incubated in vitro with molybdate, sulphide production was depressed. Molybdate was shown to inhibit only the initial rate of sulphide production in ruminal microorganisms from methionine samples in vitro. In contrast, molybdate had no effect on the rate of sulphide production in sulphate samples during the first twenty minutes of incubation. After this period, sulphide production was significantly depressed by molybdate.

Gawthorne and Nader (1976) used $^{35}$(S) sulphate to measure turnover rates of sulphate, sulphide and microbial protein-S in sheep that were intraruminally infused with 10 g/day sodium sulphate alone or in combination with sodium molybdate (50 mg Mo). The data revealed that sulphide concentrations were increased in the rumen by molybdate in vivo although there was a 50% decrease in the rate of sulphide production. Molybdate infusion did not change the amount of sulphide-S incorporated into microbial protein or affect the turnover of microbial protein.

In agreement with Huisingh et al. (1975), Gawthorne and Nader (1976) reported that a slower rate of sulphide production is due to the inhibition by molybdenum of the first sulphate reduction step, catalyzed by ATP-sulfurylase. However, Gawthorne and Nader (1976) stated that this Mo-inhibition did not lead to a lower concentration of ruminal sulphide because molybdenum also inhibits the rate of apparent absorption of sulphide from the rumen. Furthermore, these authors stated that it is erroneous to assume that the concentration of sulphide in rumen fluid is directly related to the rate of sulphide production.

Conversely, Nikolic et al. (1983) discovered that increasing the

molybdenum concentration from 0.4 to 0.61 mg/ℓ in incubated bovine rumen contents failed to alter sulphide concentration. They reported that the levels of molybdenum added to the incubation mixture were similar to those found in the rumen of sheep fed molybdenum at levels normally found in herbage (.3 to 3.5 mg/day). They concluded that, under normal feeding regimes, dietary molybdenum concentrations are not elevated enough to affect sulphide production as was shown in the in vitro experiment.

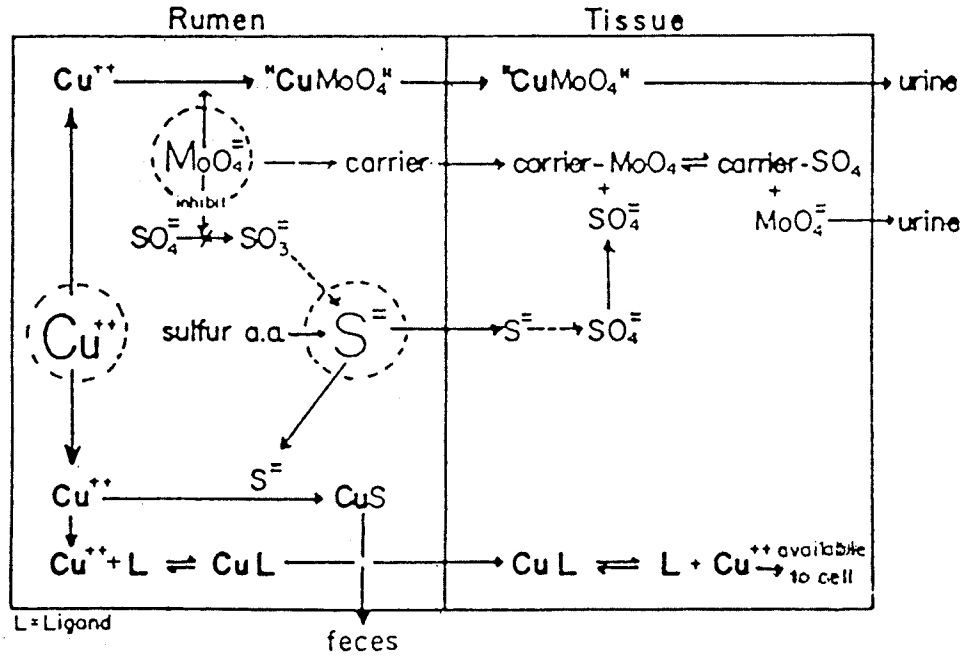
## Cu-Mo-S Interaction - Simple Working Models

Huisingh et al. (1976) developed two working models for the Cu-Mo-S interactions. In the first model, as shown in Fig. 2, copper becomes unavailable either by combining with molybdate in the rumen to form cupric molybdate or through precipitation with rumen sulphide to form insoluble copper sulphide. This model also illustrates that molybdate may either intensify a copper deficiency via cupric molybdate or alleviate it by reducing the ruminal sulphide pool.

Most studies support the model that molybdenum will reduce the sulphide pool by interfering with sulfate conversion to sulphide, by inhibiting ATP-sulfurylase activity. Therefore, molybdenum would decrease the sulphide production and thus increase the availability of copper to the animal. Huisingh et al. (1975) suggested that dietary molybdenum added to a urea-sulfate containing diet would alleviate or prevent a copper deficiency in sheep.

The model does not take into account that molybdenum interferes

Figure 2. Copper-molybdenum-sulphur interaction I. Huisingh and Matrone (1976)

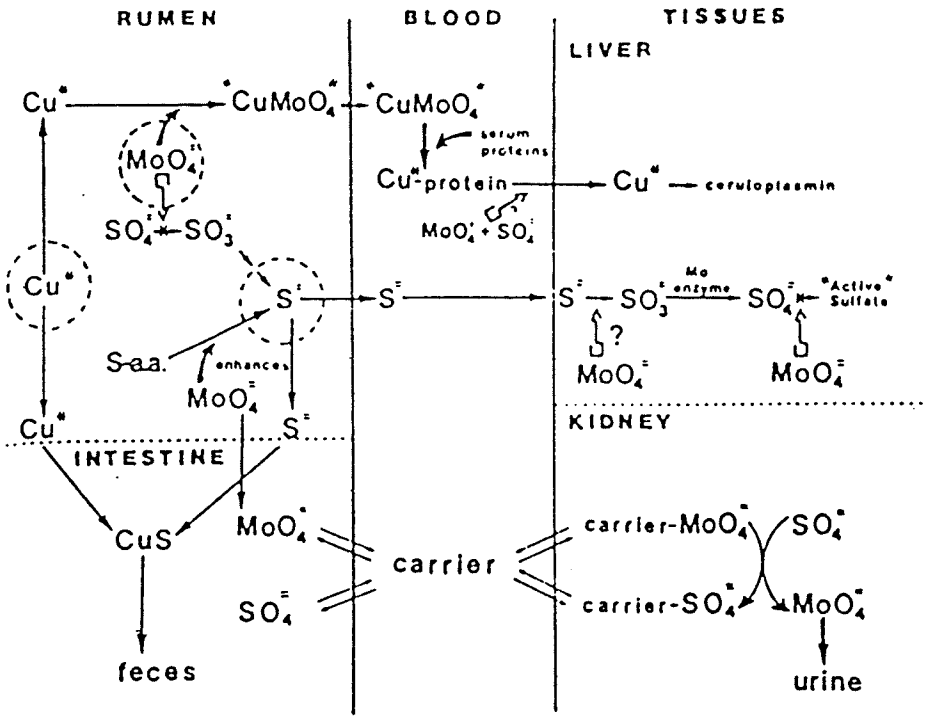


with sulphide absorption through the ruminal wall, as proposed by Gawthorne and Nader (1976). These researchers showed that molybdate infusion into the rumen actually increased sulphide concentrations because reduced sulphide absorption through the ruminal wall compensates for the decrease in the rate of sulphide reduction.

In the second model, as shown in Fig. 3, Huisingh et al. (1976), again proposed that copper becomes unavailable either by (1) formation of cupric molybdate or (2) formation of cupric sulfide. Furthermore, this model illustrates how molybdate may affect the pool of rumen sulphide, and as a result either alleviating or aggravating copper deficiency symptoms in ruminants. As shown in the first model (Fig. 2), if a diet contains sulfate as a major sulfur source, a copper deficiency may be prevented by molybdate decreasing the ruminal sulphide pool by inhibiting sulphate reduction. In contrast to the first model, the second model shows that molybdate could aggravate a copper deficiency by enhancing sulphide production from sulphur amino acids (in protein) in the diet. Huisingh et al. (1975) hypothesized that molybdate stimulated the number of $H_2S$ producing microbes, thereby increasing sulphide production in the rumen of methionine supplemented sheep.

The second model also proposes that sulphate and molybdate compete with each other for a common carrier essential for transportation across the membranes (Fig. 3). The oxy-anion (i.e. $MoO_4^{2-}$ and $SO_4^{2-}$) carrier system is thought to exist in the intestinal tract and distal tubules for reabsorption in the kidneys. Huisingh et al. (1973) pointed out that the carrier mechanism explains that with high dietary levels of molybdate and adequate dietary copper, sulphate may alleviate the adverse

Figure 3 . Copper-molybdenum-sulphur interaction II.
Huisingh and Matrone (1976)



effects of molybdate in the ruminant.

The kidney plays a large role in molybdate-sulphate interactions. Within the renal nephron, molybdate ions which normally permeate the glomerular membrane are latterly reabsorbed in the distal renal tubules, and returned to the blood. However in the presence of high levels of sulphate, the reabsorption of molybdate by the renal tubules is substantially reduced. It is hypothesized that sulphate saturates the carrier molecules of the distal renal tubules, thereby blocking molybdate reabsorption. This blockage leads to rapid molybdenum depletion via the urine (Huisingh et al. 1973).

Mason et al. (1980) demonstrated in sheep, that supplemental sulphur had no effect on the efficiency of Mo absorption, but increased the rate of Mo clearance from the blood. They accepted that since sulphate is present in macronutrient amounts, it blocks the entry of molybdate into the cells, thereby inhibiting molybdenum reabsorption from the renal tubules. These researchers also proposed that sulphur fails to block intestinal absorption of molybdenum by the same mechanism, for two main reasons. First, the capacity of the intestinal transport system may be too high to be saturated by sulphate and molybdate. Second, sulphur is absorbed as sulphide and not as a competitive sulphate.

The second model also suggests that a molybdenum/sulphate membrane carrier systems may exist in rumen microorganisms. As a result of competition with molybdate, sulphate is prevented from entering the bacterial cell. Consequently, less sulphate is converted into sulphide (Goodrich 1978). Therefore, decreased sulphide production increases the availability of copper.

Finally, the second model incorporates the influence of molybdate upon liver oxidase levels in the liver. Normally, sulphide once across the ruminal wall is transported to the liver. Sulphide oxidase in the liver catalyzes the conversion of sulphide back into sulphate which is used for metabolic purposes (Huisingh et al. 1973). Many early studies have demonstrated that liver sulphide oxidase is depressed in rats fed toxic levels of molybdenum. Siegel and Monty (1961) fed albino rats 800 mg/kg as fed Mo in a diet to study the effects of toxic levels of Mo upon sulphide oxidase activity in the liver. They found that levels of hepatic sulphide oxidase were depressed; however, the researchers attributed this enzyme decline to depressed food intake by the experimental animals caused by the Mo treatment. Underwood (1977) acknowledged that a decrease in liver oxidase activity leads to the accumulation of sulphide in the tissues. Siegel and Monty (1961) postulated that induction of copper deficiency by toxic levels of molybdate is due to the formation of insoluble copper sulphide as a result of sulphide ion accumulation in the blood and tissues.

Thiomolybdate - General Theory

Within the last decade, a concept has been developed which attempts to explain the antagonistic effects of molybdenum upon copper status in different animal species (Suttle 1980). The theory of the thiomolybdate complex and its relationship with copper provides a means to explain the complex interrelationships among copper, molybdenum and sulphur. At present, a working model of this Cu-Mo-S is not fully developed and the

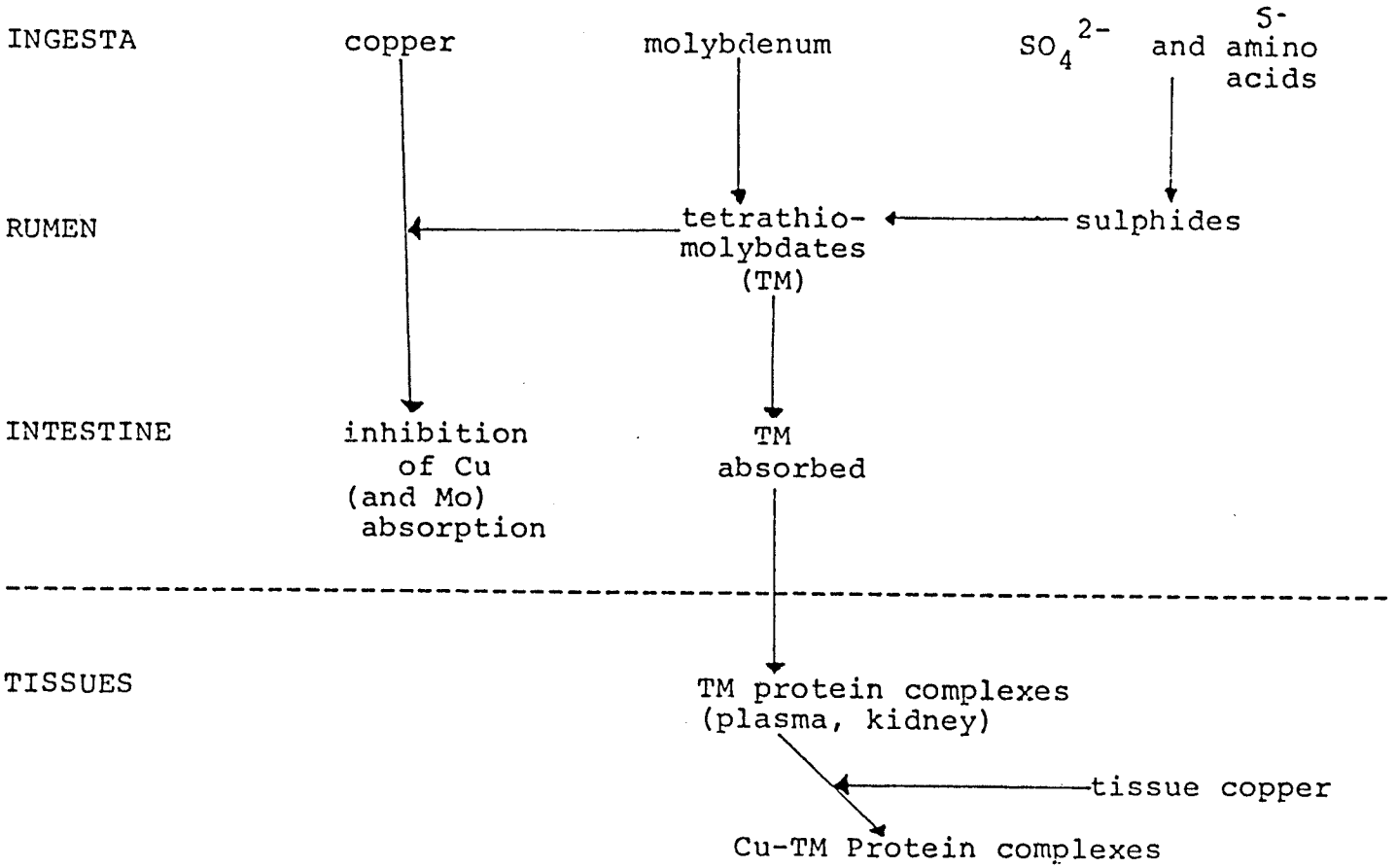
governing mechanisms are only partially understood (Lamond et al. 1980).

The thiomolybdate theory is briefly summarized in Fig. 4. Dietary molybdenum in the presence of ruminal sulphide yields oxythiomolybdates or tetrathiomolybdates in the rumen. The thiomolybdate complex combines with dietary copper in the gastrointestinal tract, thereby inhibiting copper (and Mo) absorption. As a result, a copper deficiency associated with low copper and molybdenum concentration in the tissues may occur in the ruminant (Mills 1979). Alternatively, thiomolybdates, if present in excess, may be absorbed from the digestive tract and in association with unidentified proteins, react systemically with plasma and tissue copper to produce biologically unavailable copper complexes. Such action could induce a copper deficiency in ruminants, but with Cu and Mo retained in the tissues (Mills 1979).

Thiomolybdates - Formation

Presently, the validation of the thiomolybdate theory is based upon inferences drawn from experiments in which preformed thiomolybdates were administered to ruminants, in much the same manner as dietary $MoO_4^{2-}$ + $SO_4^{2-}$ (Suttle 1980). Most of these inferences are supported by the alteration of Cu and Mo distribution by thiomolybdate's in the ruminant plasma. Similarly, the conformation of product and site of thiomolybdate formation in vivo, in the ruminant, is based upon evidence of in vitro study or from indirect evidence of in vivo experiments. The direct evidence for tetrathiomolybdate synthesis in the rumen is limited (Mason et al. 1982).

Figure 4. Mechanisms involved in the inhibitory action of dietary molybdenum and sulphur upon copper utilization by ruminants. Mills (1979)



In the 1930's, it was shown that thiomolybdates were formed in vitro when hydrogen sulphide was passed through neutral or alkaline solutions containing molybdates. These thiomolybdates were also found to form insoluble copper salts (Dick et al. 1975). Dick et al. (1975) found that a washed suspension of rumen microbes in a solution containing molybdate and sulphate led to the production of a mixture of di-, tri- and tetrathiomolybdates, identified by their absorption spectra. However, Suttle (1980) points out that spectral techniques can only detect tetrathiomolybdates in vivo at dietary Mo levels above 100 mg/kg DM (Suttle 1980).

Suttle and Field (1983) fed a molybdate derivative ($MoO_2S_2^{2-}$) to ewes to study the metabolism of thiomolybdates. They discovered that $MoO_2S_2^{2-}$ in a low S diet was adequately absorbed in the gut and did not systemically affect Cu metabolism. Such behavior was similar to $MoO_4^{2-}$ metabolism but significantly different from tetrathiomolybdate ($MoS_4^{2-}$) activity under similar low S conditions. Conversely, in a high S diet, $MoO_2S_2^{2-}$ and $MoO_4^{2-}$, like tetrathiomolybdate ($MoS_4^{2-}$) impaired Cu and Mo absorption and elevated TCA-insoluble levels of Mo and Cu in plasma. These experimenters suggested that increased rumen $S^{2-}$ concentration caused the reaction, $MoO_4^{2-} \rightarrow MoO_3S^{2-} \rightarrow MoO_2S_2^{2-} \rightarrow MoOS_3^{2-} \rightarrow MoS_4^{2-}$ to shift to the right. Therefore, the synthesis of tetrathiomolybdates in the rumen is favored under macronutrient levels of ruminal sulphide (Suttle 1980). Suttle and Field (1983) concluded that under high dietary Mo and S concentrations, enough $MoS_4^{2-}$ is produced in the rumen to interfere with Cu metabolism in the ruminant, whereas the formation of thiomolybdate derivatives (i.e. $MoO_2S_2^{2-}$) does not play an important Cu-antagonistic role.

Thiomolybdates - Absorption

Mason et al. (1978) provided evidence for thiomolybdate absorption from the gastrointestinal tract of ruminants by examining ruminant Mo absorption in vivo. They observed that duodenally administered $^{99}$Mo was rapidly absorbed and excreted via the urine. Dietary molybdenum and sulphur supplementation did not affect duodenally administered $^{99}$Mo absorption, however dietary S increased $^{99}$Mo excretion rate. On the other hand, $^{99}$Mo infused in the rumen was poorly absorbed and subsequently much of this $^{99}$Mo appeared in the feces. Dietary S supplementation in this case potentiated fecal excretion of $^{99}$Mo, whereas added molybdenum with added sulphur, improved absorption and increased urinary excretion of $^{99}$Mo. Increased absorption of $^{99}$Mo was reflected in increased amounts of $^{99}$Mo in both the soluble and insoluble plasma fractions. Consequently, TCA insoluble Cu (unavailable Cu) levels were also elevated. Conversely, absorption of $^{99}$Mo was depressed when dietary copper was ruminally supplemented along with dietary molybdenum and sulphur additives.

Mason et al. (1978) pointed out that the results of these trials support the thiomolybdate hypothesis. They postulated that dietary S is reduced to sulfide in the rumen. Sulfide in turn reacts with molybdate to form thiomolybdates. The absorption of such thiomolybdates is dependent upon the relative amounts of existing thiomolybdates and of copper in the gastrointestinal tract. In cases of thiomolybdate excess, more absorption will occur, consequently affecting copper metabolism systemically. In cases of copper excesses, the thiomolybdate is

envisioned to form insoluble complexes with copper which remain largely unabsorbed and are eliminated in the feces.

By using the technique of digesta exchange via duodenal re-entrant cannulae in sheep, Kelleher et al. (1983) also studied thiomolybdate absorption from the rumen and small intestine. $^{99}$Mo labeled tetra- and tri-thiomolybdates were rapidly (and equally) absorbed from the rumen and in these animals there was a significant rise in $^{99}$Mo serum levels, mainly in a protein bound form and TCA-insoluble. It was also found that thiomolybdates were absorbed from the duodenum of sheep receiving "pumped" digesta via re-entrant cannulae. These sheep also had elevated levels of TCA-insoluble and protein bound $^{99}$Mo as well as significantly more TCA-soluble $^{99}$Mo. These results confirm past studies that some thiomolybdates escape decomposition during acidification of digesta, and are duodenally absorbed. Furthermore, Kelleher et al. (1983) showed that infused molybdates are initially poorly absorbed from the rumen but after several hours, protein bound and insoluble $^{99}$Mo were detected in the plasma. These scientists inferred that molybdate combined with sulphide in the rumen to form absorbable thiomolybdates.

Thiomolybdates - Systemic Effects on Copper

In the ruminant gastrointestinal tract, thiomolybdates combine with copper to form unabsorbable complexes or thiomolybdates are absorbed and have systemic effects on copper (Suttle and Field 1983). In order to verify this action of thiomolybdates on copper status, especially the systemic effects, various studies have made use of several different

parameters which included TCA-soluble and insoluble Cu, plasma Cu levels, ceruloplasmin activity, and hepatic copper levels.

Suttle and Field (1983) fed a group of lambs a diet containing 3.5 mg/kg DM Cu supplemented with 3 mg/kg Mo DM ($MoS_4^{2-}$) and found that concentrations of total plasma Mo, TCA insoluble Cu and the rate of hepatic Cu depletion were increased. Likewise, Mason et al. (1978) reported that in sheep, incorporation of $^{99}Mo$ into the TCA-insoluble plasma fraction as well as elevated insoluble Cu levels occurred when dietary S and Mo were supplemented in the basal diet.

Mason et al. (1980) reported that $^{99}Mo$ administered as tetrathiomolybdate via the duodenal cannula of sheep was rapidly absorbed and caused a brief inhibition of ceruloplasmin activity (maximal over the first 2-3 h) and a decrease in TCA-soluble plasma Cu. The extent of this ceruloplasmin inhibition was directly related to the level of TCA insoluble Mo (also maximal at 2 h), such that ceruloplasmin inhibition decreased as TCA insoluble Mo levels declined. The researchers pointed out that since this reaction was so rapid, the ceruloplasmin inhibition was probably not attributable to reduced synthesis of ceruloplasmin.

In a later study, Mason et al. (1982) demonstrated that $^{99}Mo$ labelled di- and trithiomolybdates were readily absorbed after duodenal infusion in sheep. Subsequently, di- and trithiomolybdates inhibited ceruloplasmin activity and both gave rise to elevated TCA insoluble plasma Cu levels. Dithiomolybdate caused transient short term effects on ceruloplasmin activity and TCA insolubility of Cu whereas trithiomolybdates produced more prolonged effects on both.

Mason et al. (1982) suggested that initial inhibitory effects upon ceruloplasmin activity of thiomolybdate infusion are due to the direct action of co-circulating thiomolybdates. They also explain that TCA-insoluble Cu also increases because the Cu prosthetic groups of ceruloplasmin released by TCA treatment, recomplex with thiomolybdates (i.e. molybdenum trisulphide or disulphide) present in the sample and precipitate along with the proteins. Mason et al. (1982) pointed out that long term depression of ceruloplasmin is a result of thiomolybdate interference with copper metabolism. Thiomolybdates either inhibit hepatic ceruloplasmin synthesis or induce synthesis of inactive ceruloplasmin whose prosthetic groups are not released by TCA treatment and consequently are recorded as elevated TCA insoluble Cu.

Suttle (1979) focused on the effects of thiomolybdates upon hepatic Cu stores in lambs. Two week old lambs were segregated into a number of Cu and/or Mo treatment groups by supplementing copper sulphate and ammonium tetrathiomolybdate (ATM) in a milk replacer and later in a pelleted diet. The results showed that thiomolybdates did not greatly accelerate the removal of Cu accumulated in the liver. Suttle (1979) concluded that ATM interferes with Cu absorption from the gut rather than by systemic action.

## Conclusion

Although the presence of copper in the body was discovered in the early 19th century, copper has been recognized as an essential dietary trace element for animals in the last 50 years. Copper is widely distributed throughout the body, most of which is concentrated in the liver.

Copper is an important constituent in many metalloproteins and enzyme systems. As an essential component of ceruloplasmin, copper plays a central role in the utilization of iron in the animal body. Similarly, copper, as part of monoamine oxidase is involved in the crosslinkage and maturation of collagen. Such copper parameters as liver Cu, serum Cu and plasma ceruloplasmin activity are widely utilized to measure copper status in ruminants, but which criterion is the best indicator is highly controversial.

An exact copper requirement for the ruminant has not been defined, however recommended dietary levels have been established to maintain good health in the animal, the specific levels being influenced by a variety of factors. Primary copper deficiency occurs in ruminants when the diet does not contain an adequate copper level to meet dietary requirements. Clinical symptoms of a copper deficiency in cattle range from a simple loss of coat color to anemia and bone disorders. In more severe copper deficiency cases, death of the animal may result. Secondary or conditioned copper deficiency is induced in ruminants when elevated molybdenum levels in the diet reduce the utilization of dietary copper in the animal such that the copper requirement cannot be achieved. Furthermore, a high sulphur level in the same diet may potentiate the adverse effect of molybdenum.

There is a proven antagonistic interrelationship between copper, molybdenum and sulphur in the ruminant. Although several hypotheses have been developed, the understanding of the mechanisms of this triple interaction, remains unclear and incomplete. These mechanisms probably include: (1) formation of an unabsorbable Cu-Mo complex, (ii) formation

of an insoluble Cu-S complex and (iii) competition for a common carrier system involving Mo and S.

Recently, a fourth mechanism has been postulated which offers a more complete explanation of this three-way interaction, namely, the thiomolybdate theory. This thiomolybdate theory suggests that copper is rendered unavailable in two ways: thiomolybdates are synthesized in the rumen; which leads to the formation of insoluble Cu-Mo-S complexes in the gut and consequent reduction in copper absorption, or thiomolybdates are formed in the rumen and are absorbed from the gut. These thiomolybdates form biologically unavailable complexes with copper in the blood and tissues, thus producing systemic effects on copper metabolism.

In the last ten years, the three-way interaction between Cu, Mo and S has been the focus of much study. Surveys have been performed in Northwestern Manitoba to investigate the extent of a secondary copper deficiency of beef cattle grazing pasture. Many experiments have been established under feedlot conditions to study the effects of molybdenum and sulphur on copper status of ruminants. Additional study has examined the use of copper supplementation (i.e. via diet or injection) to alleviate primary and secondary deficiencies in ruminants. Data from this present research, would suggest that the interrelationship between Cu, Mo and S is very complex and that the sole action of one mineral element cannot be considered alone.

Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on the copper status of beef steers fed for 121 days.

## EXPERIMENTAL OBJECTIVES

1. To study the effects of four different concentrations of Cu and Mo at a 2:1 ratio in the diet of yearling beef steers on:
   a. changes in liver Cu, total serum copper, ceruloplasmin activity (a Cu dependent enzyme) and Trichloroacetic acid (TCA)-soluble Cu levels.
   b. progressive changes over 121 days on a monthly basis in liver and serum copper parameters.
2. To examine the interrelationships between liver copper and blood copper.
3. To determine which of the criteria being evaluated was the best for assessing copper status in beef cattle fed high Mo diets.
4. To examine dietary effects on animal live weight gain, dressed carcass weight and carcass grade.

## MATERIALS AND METHODS

### Animals and Management

Sixteen yearling beef steers were used in a split-plot design with repeated measurements over time. Twelve of the steers used in the experiment were Selkirk-reds. This strain was developed at the University

of Manitoba based on the Charolais, Angus and North Devon breeds of cattle. The remaining four cattle were commercial Hereford cattle.

At the beginning of the experiment, the sixteen steers were allotted to four treatment groups (4 per pen) equalized on the basis of breed and weight. Three Selkirk-red cattle and one Hereford were assigned to each pen. All animals were housed in a poll barn open to the south with half of the pen outside and the other half sheltered. Each pen contained a feed bunk and an automatic waterer. Straw bedding was provided on the concrete floor of each pen.

About two weeks into the experiment, all animals received an intramuscular injection of a preparation of vitamins A, D and E.

Design and Diet

For a total of 121 days, each pen of four steers received approximately 36 kg daily of a complete feed based on barley and alfalfa-bromegrass hay plus a premix. This pelleted basal diet was formulated to meet NRC (1976) requirements for growth at 0.9 kg/day (Table 1). Copper oxide (CuO) and ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) were supplemented in the premix in order to provide the various concentrations on a dry matter basis of Cu and Mo at a 2:1 ratio in the diet. The treatment groups were as follows: (A) 7.0 ppm Cu:3.5 ppm Mo, (B) 15 ppm Cu:7.5 ppm Mo, (C) 30 ppm Cu:15 ppm Mo and (D) 100 ppm Cu:50 ppm Mo. Table 2 shows the analysed composition of the treatments.

The first week of the experiment was designated as an adjustment period, so that the cattle could adjust to the pelleted diets.

Table 1. Experiment 1. Composition of basal and treatment diets fed to beef yearling steers for 121 days

| Composition | Basal diet |
|---|---|
| Alfalfa-bromegrass hay (%) | 42.5 |
| Barley (%) | 55.0 |
| Premix (%) | 2.5 |
| Urea (%)[ab] | 0.5 |
| NaCl (%)[ac] | 0.5 |
| ZnO (mg/kg)[ad] | 41.3 |

| | Treatments | | | |
|---|---|---|---|---|
| | A | B | C | D |
| CuO (mg/kg)[ae] | 1.58 | 12.0 | 31.7 | 123.3 |
| $NH_4MoH_2O$ (mg/kg)[af] | 4.40 | 11.6 | 25.1 | 88.0 |

[a] Ingredients listed under premix (including CuO and $(NH_4)_6Mo_7 \cdot 4H_2O$ are supplemented in the premix of the diet. The amounts of these ingredients are recorded as the level in the complete diet.

[b] Urea = 45% N.

[c] Cobalt-iodized salt.

[d] ZnO = 72.0% Zn

[e] CuO = 75.0% Cu.

[f] $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ = 54.3% Mo.

Table 2. Experiment 1. Chemical composition (analyzed) of treatment diets (dry matter basis) fed to beef steers

| | | | Treatment | | | |
|---|---|---|---|---|---|---|
| | Hay | Barley | A | B | C | D |
| Nitrogen (%)[a] | 2.29 | 1.67 | 2.16 | 2.14 | 2.16 | 2.16 |
| Protein (Nx6.25) (%)[a] | 14.3 | 10.4 | 13.5 | 13.5 | 13.5 | 13.5 |
| ADF (%)[a] | 30.4 | 10.9 | 17.4 | 17.4 | 17.4 | 17.4 |
| Energy (KJ/g)[a] | 17.4 | 16.9 | 17.0 | 17.0 | 17.0 | 17.0 |
| Dry matter (%) | 87.0 | 88.9 | 91.5 | 92.5 | 90.8 | 92.5 |
| Ca (%) | 1.55 | 0.05 | 0.55 | 0.56 | 0.53 | 0.60 |
| P (%) | 0.23 | 0.45 | 0.32 | 0.36 | 0.35 | 0.36 |
| S (%) | 0.19 | 0.17 | 0.22 | 0.20 | 0.20 | 0.18 |
| Mg (%) | 0.32 | 0.13 | 0.20 | 0.21 | 0.20 | 0.23 |
| Zn (mg/kg) | 34.9 | 26.4 | 51.1 | 52.2 | 48.7 | 60.4 |
| Fe (mg/kg) | 59.6 | 48.4 | 69.3 | 89.4 | 76.4 | 67.9 |
| Mn (mg/kg) | 24.0 | 8.6 | 19.0 | 19.3 | 18.0 | 18.5 |
| Cu (mg/kg) | 7.8 | 4.5 | 7.9 | 16.0 | 26.0 | 110.4 |
| Mo (mg/kg) | 1.1 | 1.2 | 3.6 | 7.2 | 15.3 | 61.1 |
| Cu:Mo ratio | 7.1 | 3.8 | 2.2 | 2.2 | 1.7 | 1.8 |

[a]Representative sample of all 4 treatments was obtained and thoroughly mixed together for analysis for protein, ADF, energy content.

Several mixes (250 kg each) of each treatment were produced on three separate occasions. Samples of each diet on each occasion were taken.

Sampling Procedure

All animals were weighed and liver biopsies obtained approximately every 30 days (first week of the month) and jugular blood samples were taken on a weekly basis.

The liver biopsy technique was similar to a procedure used by Erwin et al. (1956). After the animal was restrained in a squeeze-chute, the hair was clipped over the 11th, 12th and 13th ribs on the right side of the animal. An incision was made with a scalpel at the 11th intercostal space, such that a liver biopsy sample could be obtained with the biopsy trocar instrument. Immediately, after the sample was removed from the animal, the piece of liver was blotted on paper to remove excess blood and placed in 2 dram vials and frozen at $-20^{o}C$ until analysed. Liver samples were typically 2.0 to 5.0 cm in length and weighed 0.2 to 0.7 grams.

To protect against infection, certain precautions during liver biopsying were implemented. All biopsy instruments and scalpel were placed in cold sterilization solution between samplings. The shaved area on each steer was washed with a solution of water and surgical skin soap (Savlon) prior to surgery. After the liver sample was obtained, the wound was sprayed with an antiseptic. After the third sampling period, a standard practice of injecting each animal with 10 cc of penicillin a half hour prior to surgery was also established.

At the end of the experiment, all animals were slaughtered and liver samples (approximately 1 kg) were obtained at the abattoir. In the lab, a strip, approximately 2-3 cm in length was taken from each liver sample and treated in a similar manner to previously obtained biopsied liver samples.

Blood samples were obtained from the jugular vein from each animal using 15 ml vacutainer tubes. These blood filled vacutainers were placed in a cooler overnight to allow the blood to clot. Serum was obtained upon centrifugation of the clotted blood and placed in 2 dram vials. All serum samples were frozen at -20$^{o}$C until analysed. Subsequently, these serum samples were analyzed for copper content and ceruloplasmin activity.

Sample Determinations

All seventy-four biopsy samples collected throughout the experiment were analyzed for copper concentration. Of the weekly serum samples only a portion were analyzed for copper content. The determination of copper content in serum samples at the time of liver biopsy (on a monthly basis) were reported in accordance with experimental objectives 1a and 1b of this study. Intermediate serum samples obtained on a weekly basis were mainly utilized for the practice and refinement of procedures used to determine ceruloplasmin activity in serum. However, like serum Cu data, ceruloplasmin assay results are reported on a monthly basis at the time of liver biopsy.

The yield of serum from many of the jugular blood samples taken at the time of liver biopsy was adequate for both a serum Cu and ceruloplasmin analysis but not for a TCA-soluble Cu analysis. Therefore, it

was decided to determine the TCA-soluble Cu content in serum samples acquired at a point closest to the time of liver biopsy. Serum samples acquired 3 days after biopsy (except for the last period which represents 3 days before biopsy) were appropriately chosen. The level of serum Cu and ceruloplasmin activity were also determined in these samples in order to establish the association between these two serum parameters and TCA-soluble Cu. The behavior of serum Cu and ceruloplasmin activity was also compared to their counterparts at the time of liver biopsy in terms of the effects of treatment and period.

Analytical Techniques

Liver and serum samples were prepared by a modified wet ashing method of Thompson and Blanchflower (1971) for determination of the copper content of the sample by atomic spectrophotometry. To 6 dram screw-cap vials containing a liver sample or 2 ml of serum, 3 ml of a nitric:-perchloric acid (4:1 v/v) mixture was added and allowed to remain at room temperature overnight. After this predigestion phase, the vials were moved to an aluminum block which was custom made to enclose the lower half of the vials when placed on top of a block heater. After several hours of digestion as described by the method used, the ashed samples were diluted with 5% HCl and the resulting solution was aspirated into the atomic absorption spectrophotometer (Instrumentation Laboratory Model 551) for Cu analysis.

Standard solutions were made by diluting a commercial standard solution (1000 ppm, Cu) with 5% HCl to the desired concentrations and aspirated into the atomic absorption spectrophotometer.

Before being used for copper determination, all glassware was washed with soap and water and rinsed in de-ionised single distilled water. Furthermore, the glassware was then placed in a 5-10% $HNO_3$ (in de-ionized water) bath for 16-24 hours. After removal, all glassware was dried in a heated oven.

Serum ceruloplasmin was determined by the method described by Rice (1963) with slight modifications which measure the enzyme's oxidative activity toward p-phenylenediamine (PPD). The pH optimum for cattle p-phenylenediamine oxidase activity was determined as 6.4 (Bingley and Dick 1969) and this value was used in the procedure rather than the pH of 5.2 used by Rice (1963). This procedure spectrophotometrically measures the oxidation product of 0.10% PPD at 540 mμ, 37°C and pH 6.4, in acetate buffer with EDTA (ethylenediaminetetraacetate). The determinations were done in duplicate using serum blanks. Four samples were analyzed at a time in order to reduce error. Enzyme activity was expressed in terms of arbitrary absorbance units (540 mμ) at the end of a 15 minute incubation period.

An alternative means was investigated to report ceruloplasmin activity in quantitative terms other than absorbance units at 540 mμ. A modified procedure by Rice (1962) was employed to express bovine ceruloplasmin activity in International Enzyme Units (IEU). A standard curve of absorbance at 540 mμ versus the concentration (μg/ml) of standard solutions of Bandowski's base (substituting human sera used by Rice (1962) with bovine sera) at 0 and 2 hours after preparation and their respective slopes were determined (Fig. 5). The slope of the calibration curve at t = 0 hr was used in an equation developed by Rice (1962) to

Figure 5. Bandowski's Base: Calibration curve to determine International Enzyme Unit constants at 0 and 2 hours after preparation of standard stock solutions.

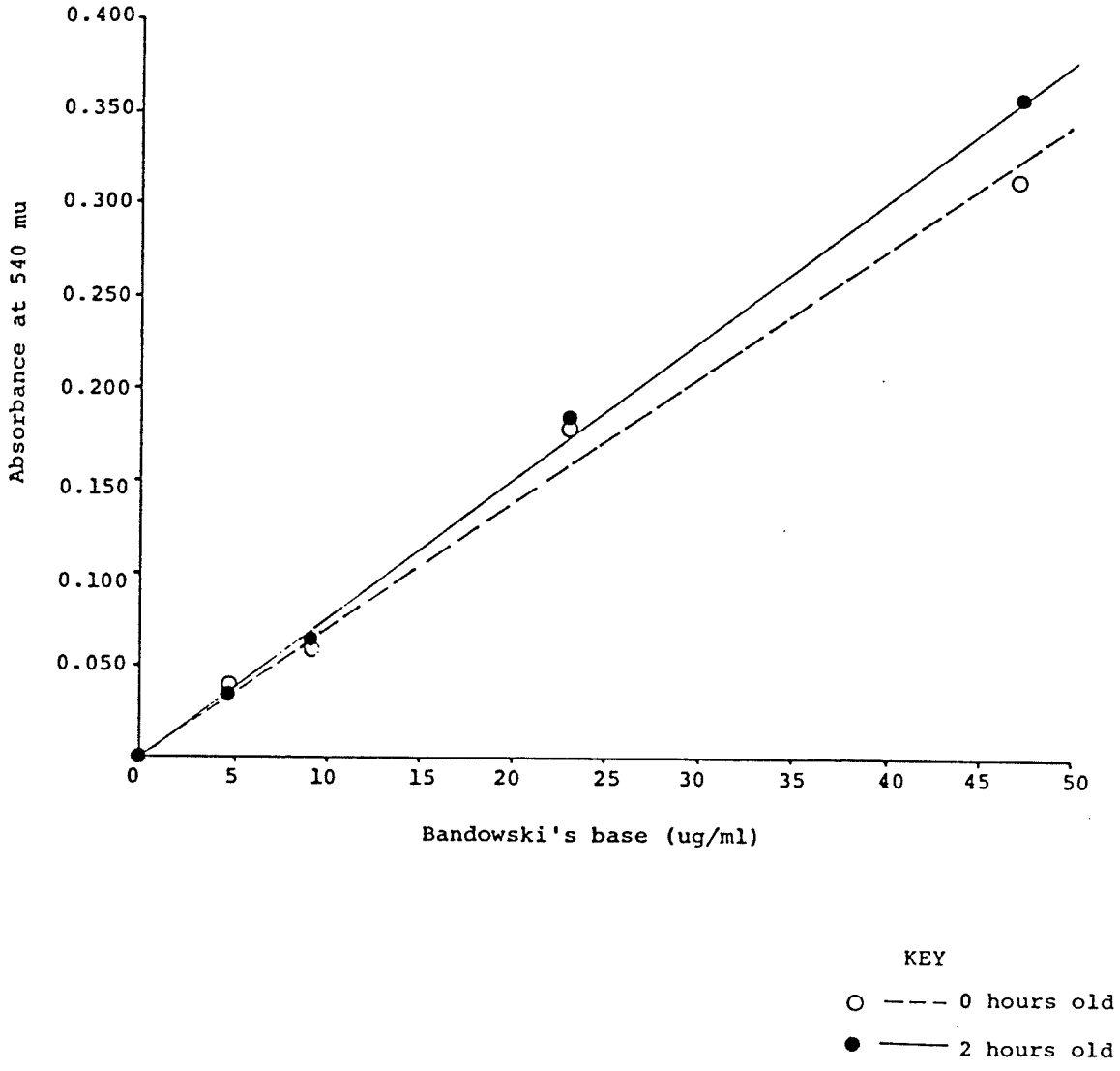


Table 3. Bandowski's Base: Abs. at 540 mμ to determine International Enzyme Units (IEU) constants at 0h and 2h after preparation of stock solution

Stock sol'n: 0 hours old

| Std. conc. (μg/ml) | Actual conc. (μg/ml) | Abs. at 540 μm |
|---|---|---|
| 0.0 | 0.0 | 0.000 |
| 5.0 | 4.7 | 0.041 |
| 10.0 | 9.4 | 0.060 |
| 25.0 | 23.5 | 0.171 |
| 50.0 | 47.0 | 0.320 |

Stock sol'n: 2 hours old

| Std. conc. (μg/ml) | Actual conc. (μg/ml) | Abs. at 540 μm |
|---|---|---|
| 0.0 | 0.0 | 0.000 |
| 5.0 | 4.7 | 0.036 |
| 10.0 | 9.4 | 0.064 |
| 25.0 | 23.5 | 0.178 |
| 50.0 | 47.0 | 0.355 |

Table 4. Bandowski's Base: Calculations to determine International Enzyme Units (IEU) constants at 0h and 2h after preparation of stock solutions

$$IEU = (A/B)\ (10{,}000/15) = A(C)$$

A = Absorbance of serum ceruloplasmin assay, 540 µm

B = Slope of standardization curve x MW

C = Multiplication factor

MW = Molecular weight of Bandowski's base

Stock sol'n: 0 hours old

$$I.E.U. = \frac{A}{2.165} \times \frac{10000}{15} = A(307.93) \qquad C=307.93$$

Stock sol'n: 2 hours old

$$I.E.U. = \frac{A}{2.413} \times \frac{10000}{15} = A(276.28) \qquad C=276.28$$

establish a multiplication factor (C) which converts ceruloplasmin activity from absorbance units into IEU (Table 4). An additional multiplication factor derived from the slope at t = 2 hr was determined to investigate the effects of stock solution shelf life upon the slope of the calibration curve. Rice (1962) pointed out that absorbance of the stock solution should be determined within one hour of preparation. After this period the color intensity of the solutions fade and thus alters spectrophotometric readings.

From the standardization technique using standard solutions of Bandowski's base (with bovine serum), it was found that the multiplication factor needed to convert arbitrary ceruloplasmin activity units to IEU was equal to 307.93. If the standard solutions were allowed to stand for two extra hours this multiplication factor fell to 276.28 (Fig. 5).

An attempt was made to test the purity of the synthesized Bandowski's base by measuring the melting point of the crystals. Pure Bandowski's base crystals have a sharp melting point of 238-240$^{o}$C. By using a melting point apparatus it was found that synthesized crystals turned black and charred at 200-215$^{o}$C. Identical results were found upon duplication.

In the present study, arbitrary units (in terms of absorbance at 540 mμ) were chosen to express ceruloplasmin assay results rather than IEU as proposed by Rice (1962). Solutions of Bandowski's base are derived from oxidized p-phenylenediamine (PPD) free-form crystals and not dihydrochloride PPD as used in ceruloplasmin assays. Free-form PPD and

dichloro-PPD are two different compounds. Therefore, a multiplication factor (for IEU) established for dichloro-PPD may differ from a multiplication constant characteristic of PPD free-form. Furthermore Bandowski's base could not be made with dihydrochloro-PPD.

The TCA-soluble copper fraction of serum was recovered by treating the serum sample with 10% and 5% trichloracetic acid solutions as outlined by Mason et al. (1978). Acid washed disposable culture tubes (12x75 mm) were used for recovery of supernatant, the TCA-soluble Cu fraction. The TCA-soluble fractions were analyzed for copper by atomic absorption spectrophotometry. A weight of recovered supernatant was corrected to a volume basis by weighing a known volume of 5% TCA to obtain a weight/volume correction factor. The correction factor was used to convert the weight of recovered supernatant to the appropriate recovered volume.

Standard solutions were made by diluting a commercial standard solution (1000 ppm Cu) with 5% TCA to the desired concentrations and aspirated into the absorption spectrophotometer, accordingly. Before any analysis was performed on feed samples obtained from feed bags, all pellet samples were ground through a 1 mm stainless steel screen in a Wiley hammer mill. Similar to liver and serum determination, feed samples (0.2-0.4 g) were wet ashed and analyzed for Cu and Zn content by the atomic absorption spectrophotometer. Likewise, the Mo content of a feed was analyzed in a similar manner after wet ashing a representative sample and diluting it in 5% HCl with 1000 ppm added $Na_2SO_4$. Furthermore, Cu, Zn, Mn, Fe, Cu and Mg concentrations of the diets were determined by dry ashing 1 g of each feed sample and diluting with 5N

HCl. The fixed solutions were aspirated into the atomic absorption spectrophotometer and the respective mineral concentrations were read. The procedures were in compliance with those developed by the Association of Official Analytical Chemists (A.O.A.C. 1980).

The level of sulphur in the diets was evaluated by a modified procedure for the determination of total sulphur in plant material based on Hamm et al. (1973). One-half sample of pelleted diet was digested with nitric acid-perchloric acid in a microkjeldahl flask and boiled to dryness. The residual matter containing the sulphur as sulphate was dissolved in three successive washings of distilled water. Each washing was filtered through Whatman #42 (ashless) filter paper into a 25 ml volumetric flask. An aliquot of this solution (0.40 ml) was transferred to 10 ml 0.001M calcium chloride for sulphur determinations by a Technicon analyzer.

Phosphorus in feed was analyzed by a photometric method of A.O.A.C. (1975).

Feed samples were also analyzed for dry matter, crude protein, acid detergent fiber and energy as described by the A.O.A.C. (1980).

## Statistical Analysis

Repeated measurements of cattle body weight, liver Cu, total serum Cu (regardless of weekly obtained blood samples), ceruloplasmin activity and TCA-soluble Cu levels were taken at the beginning and at subsequent monthly intervals of the experiment for all treatment groups. A split-plot design was utilized to test the significance ($P<.05$, $P<.01$) of the main effects of treatment and period and their treatment x period

interactions on changes in the level of copper criteria (excluding weight gain results) from time zero data. To calculate this change, the level of each criteria at the end of each period was subtracted by respective initial values at the beginning of the experiment. This change is symbolized as $\Delta_1$.

Likewise, a Tukey's multiple range test was also used to test differences among treatment groups with respect to mean overall changes in cattle body weight gain, liver Cu, total serum Cu and ceruloplasmin activity. These overall changes were found by subtracting initial values at the beginning of the experiment from final values at the end of period IV. This overall change is symbolized as $\Delta_2$. If significant differences ($P<.05$) arose, Tukey's test showed which overall ($\Delta_2$) means among the treatment groups were significantly different and which ones were not.

Correlation coefficients between liver Cu, serum Cu, ceruloplasmin activity and TCA-soluble Cu levels were determined by simple linear regression. A test of significance of the difference between two correlation coefficients was also utilized.

## RESULTS

Live weight gain, average daily gain (ADG) and dressed carcass weight among steers did not differ significantly ($P>.05$) among treatment groups as shown by Tukey's multiple range test (Table 5). The ADG among treatment groups ranged from 1.07-1.16 kg/day. At slaughter, the percentage of dressed carcass yield from all steers ranged from 49-54% and averaged 51.68% over all treatments. All animals graded A1-A2

Table 5. Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on weight gain ($\Delta_2$) in beef steers fed for 121 days[1]

| Treatment[2] | Live weight gain (kg.) | ADG[3] | % dressed carcass wt. |
|---|---|---|---|
| 7.0 ppm Cu: 3.5 ppm Mo | 130.0[a] | 1.07[a] | 50.75[a] |
| 15.0 ppm Cu: 7.5 ppm Mo | 132.0[a] | 1.09[a] | 51.72[a] |
| 30.0 ppm Cu: 15.0 ppm Mo | 140.0[a] | 1.16[a] | 51.52[a] |
| 100.0 ppm Cu: 50.0 ppm Mo | 132.7[a] | 1.10[a] | 52.74[a] |

[1]Values within columns with different subscripts are significantly different ($P<.05$).

[2]Treatment groups are referred to in text as Groups A, B, C, D, respectively (in order of increasing Cu, Mo concentration in treatment).

[3]ADG = Average daily gain (kg/day), 121 day basis.

except for two animals which were judged B1's.

Changes in liver Cu levels of steers as shown by split-plot analysis were significantly ($P<.01$) affected by treatment (Table 11A). Copper content in the liver increased over the four periods ($P<.01$), however there was no treatment x period interaction ($P>.05$) in the experiment (Table 11A). Tukey's multiple range test (Table 6) and Figure 6 indicate that the steers on the treatment of highest concentration of Cu and Mo (Group D; 100 ppm Cu:50 ppm Mo) had the greatest increase in liver Cu (+141.57 ppm) over the four month period. To a lesser magnitude, significant ($P<.01$) increases were recorded in the second (+67.89 ppm) and third (+76.47 ppm) treatment groups (Groups B and C). The rise in liver Cu recorded for the first group (Group A; 7.0 ppm Cu:3.5 ppm Mo) was the smallest of all four treatment groups (+33.89 ppm).

The level of total copper in serum (Fig. 7) was significantly ($P<.05$) affected by period (Table 12A). Changes in serum Cu were not significantly ($P>.05$) affected by treatment (Table 12A); however, with the exception of group A, which recorded a small loss in serum Cu (-4.8 µg/ml) each of the other treatment groups had overall gains in total serum Cu (Table 6, Fig. 7) by the end of the experiment. In addition, serum Cu was not significantly ($P>.05$) affected by treatment x period interactions.

Serum ceruloplasmin activity was not significantly ($P>.05$) affected by dietary treatment or treatment x period interaction (Table 13A). However, whereas groups A and B had changes in ceruloplasmin activity of 0.005 (arbitrary units - absorbance at 540 µm), groups C and D had changed ceruloplasmin activities of 0.025 and 0.033 respectively

Table 6. Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on changes ($\Delta_2$) in liver Cu, serum Cu and ceruloplasmin activity in beef steers fed for 121 days[1]

| Treatment[2] | Liver Cu ($\Delta_2$)[3] | Serum Cu ($\Delta_2$)[3] | Ceruloplasmin activity ($\Delta_2$)[4] |
|---|---|---|---|
| 7.0 ppm Cu: 3.5 ppm Mo | 33.89[a] | -4.8[a] | .005[a] |
| 15.0 ppm Cu: 7.5 ppm Mo | 67.89[ab] | 9.8[a] | .005[a] |
| 30.0 ppm Cu: 15.0 ppm Mo | 76.47[b] | 3.6[a] | .025[a] |
| 100.0 ppm Cu: 50.0 ppm Mo | 141.57[c] | 5.8[a] | .033[a] |

[1]Values within columns with different superscripts are significantly different ($P<.05$).

[2]Treatment groups are referred to in text as Groups A, B, C, D, respectively (in order of increasing Cu, Mo concentration in treatment).

[3]mg/kg of Cu in liver, wet basis and µg/100 ml of Cu in serum.

[4]Absorbance measured at 540 mµ.

Figure 6. Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on LIVER Cu of beef steers fed for 121 days.

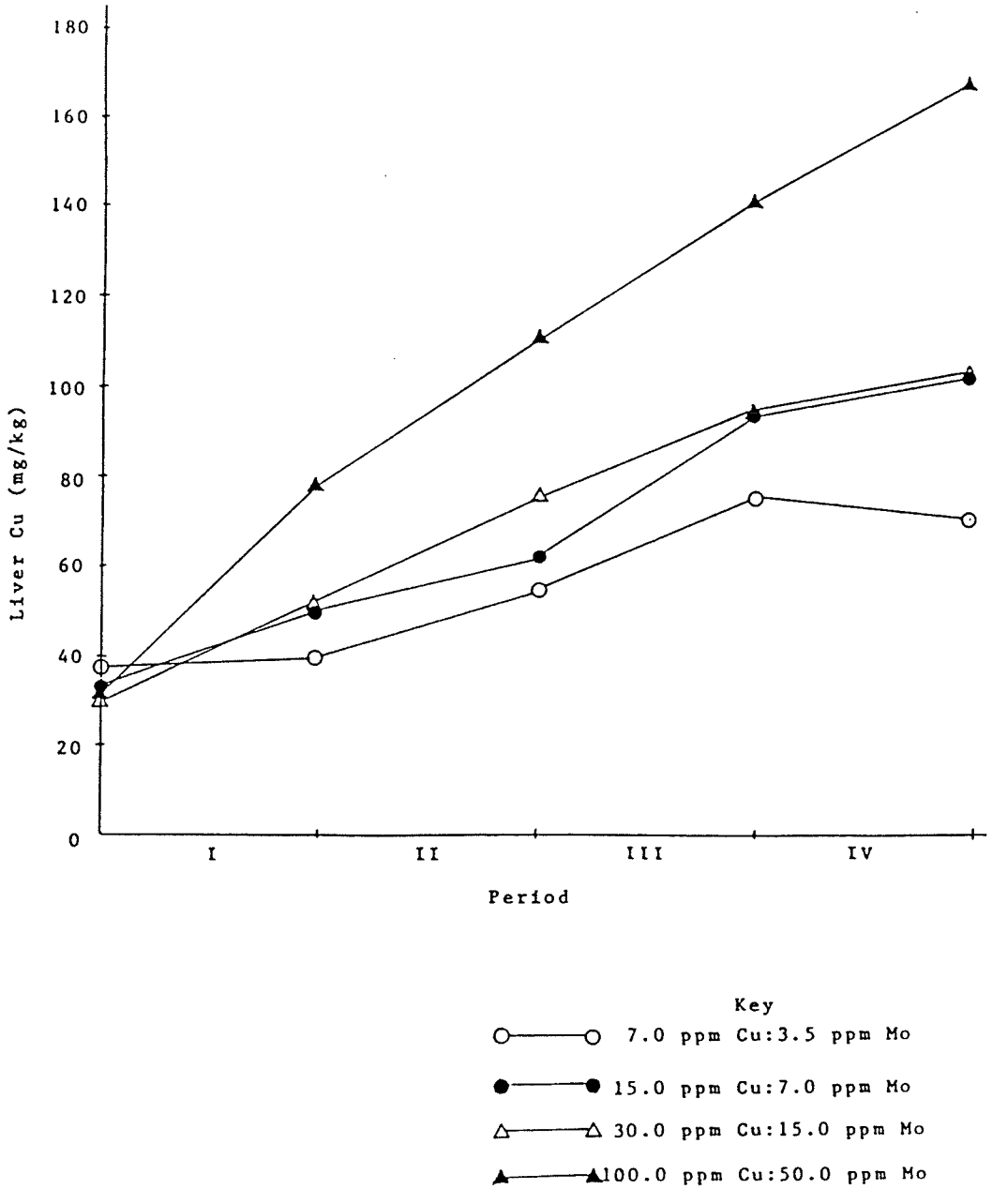
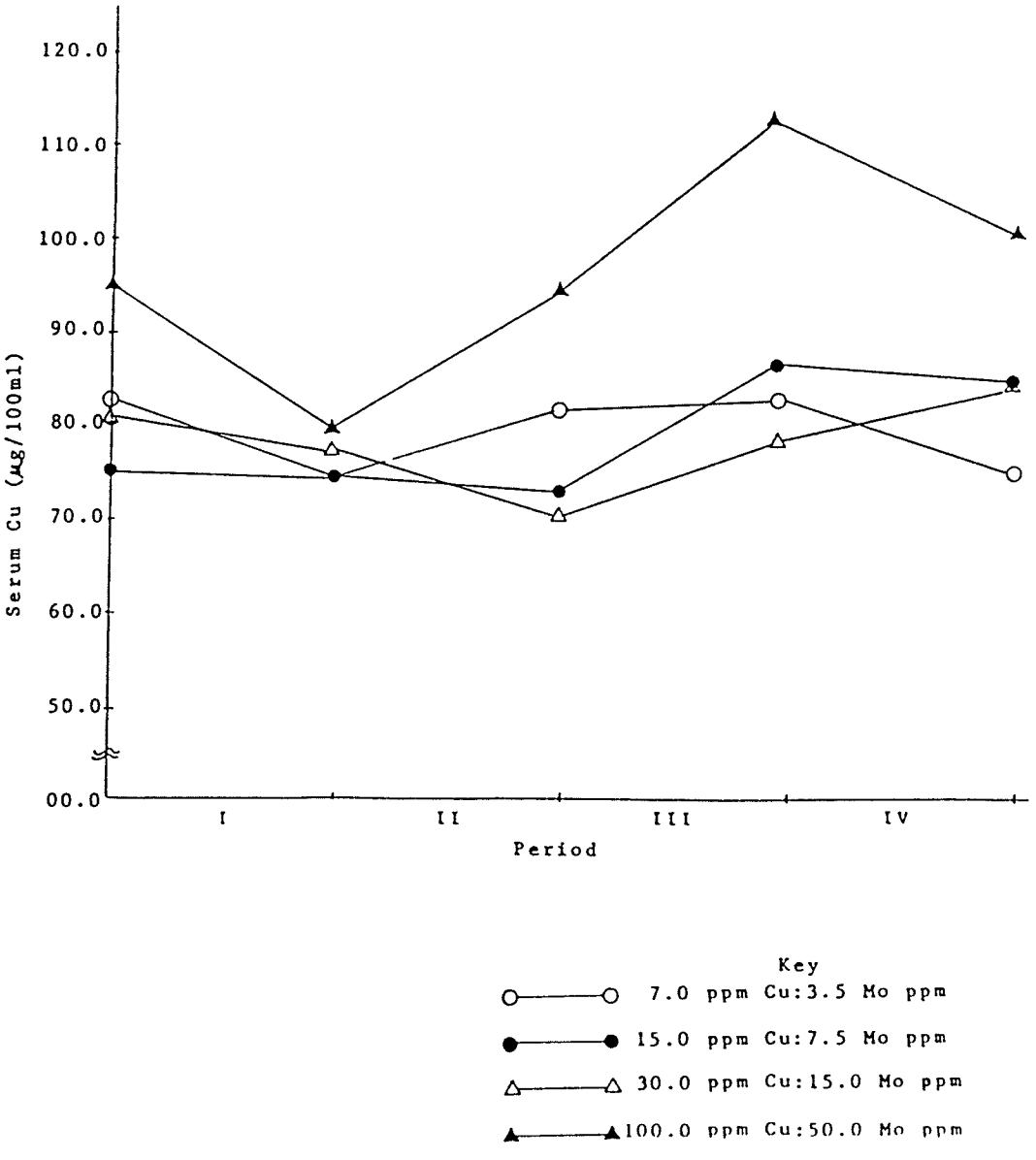


Figure 7. Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on SERUM Cu of beef steers fed for 121 days.



(Table 6, Fig. 8). Furthermore, ceruloplasmin activity among the cattle was significantly ($P<.01$) affected by period (Table 13A). Although, ceruloplasmin activity was depressed among all four pens at periods throughout the experiment (Fig. 8), all treatment groups finished the experiment with small but positive changes in ceruloplasmin activity (Table 6).

Serum Cu content and ceruloplasmin activity of serum samples obtained 3 days after biopsy reacted to the effects of treatment and period in the same manner as their counterparts at the time of liver biopsy with respect to split-plot analysis (Table 14A and 15A). TCA-soluble Cu levels (derived from the same serum samples) among treatment groups were not significantly ($P>.05$) affected by treatment or period (Table 16A). The level of TCA-soluble Cu in total serum Cu (obtained 3 days after biopsy) ranged from 84-98% (Fig. 9). Compared to initial values, TCA-soluble Cu content in total serum Cu in groups A, B and C by the end of the experiment remained unaltered (±1.5%). In contrast, animals on treatment D (100 ppm:50 ppm Mo) showed a decrease of 13.68% of TCA-soluble Cu in total serum Cu.

The relationships among liver and blood copper parameters (at the time of liver biopsy) were examined by correlation coefficients (Table 7). The correlation coefficient between liver Cu and total serum Cu was low, $r = .37$. This value indicates that only 13.7% ($r^2 \times 100$) of the variation in serum Cu was related to changes in hepatic concentration. The correlation coefficient between total serum Cu and serum ceruloplasmin activity was also low, $r = .37$. Hepatic Cu had a correlation coefficient of $r = .23$ with serum ceruloplasmin.

Figure 8. Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on CERULOPLASMIN ACTIVITY of beef steers fed for 121 days.

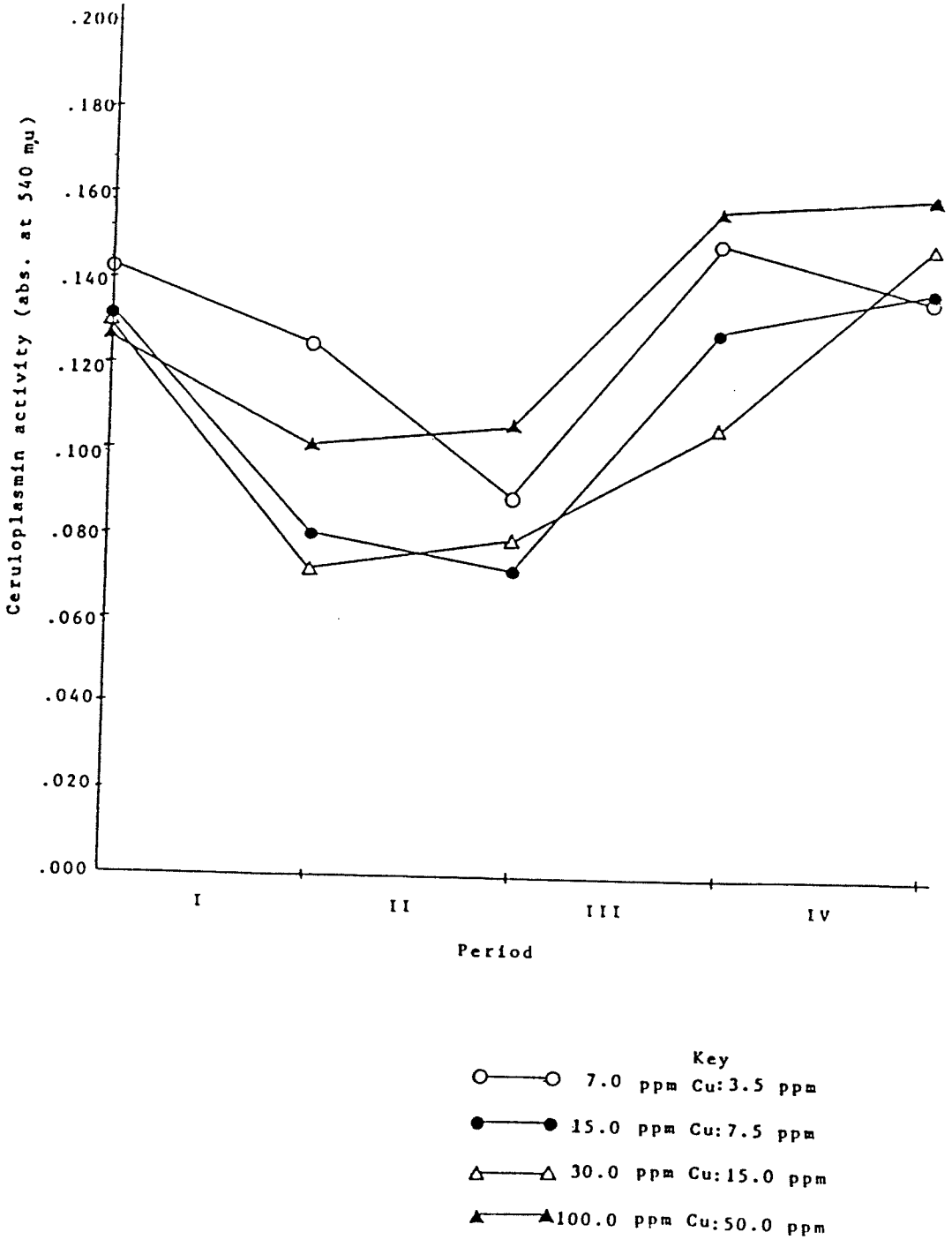
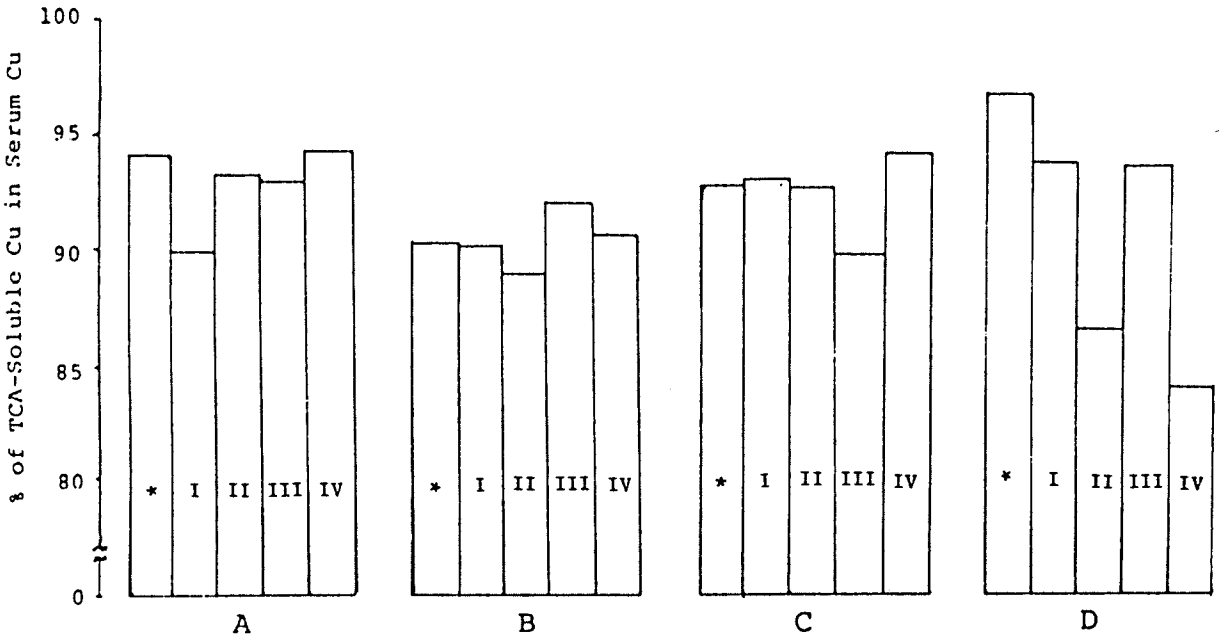


Figure 9. Experiment 1. The effect of different concentrations of copper and molybdenum in a 2:1 ratio in the diet on the % of TCA-SOLUBLE Cu of beef steers fed for 121 days.




Key

A = 7.0 ppm Cu: 3.5 ppm Mo
B = 15.0 ppm Cu: 7.5 ppm Mo
C = 30.0 ppm Cu:15.0 ppm Mo
D =100.0 ppm Cu:50.0 ppm Mo

* = initial period
I = period I
II = period II
III = period III
IV = period IV

Table 7. Experiment 1. Correlation coefficients among Cu criteria (at biopsy) of beef steers[1-4]

| Y/X | Liver Cu | Serum Cu | Ceruloplasmin |
|---|---|---|---|
| Liver Cu | 1.00 | .37 | .23 |
| Serum Cu | - | 1.00 | .37 |
| Ceruloplasmin | - | - | 1.00 |

[1]N = 74

[2]X = independent variable and Y = dependent variable.

[3]Serum Cu (sampled at liver biopsy).

[4]Ceruloplasmin, from serum samples at biopsy.

The correlation coefficients between respective serum parameters (3 days after biopsy) are shown in Table 8. The correlation coefficient between TCA-soluble Cu and serum Cu (total) was very high; r = .94. Total serum Cu had a correlation coefficient of r = .62 with ceruloplasmin activity. TCA-soluble Cu had a correlation coefficient of r = .59 with ceruloplasmin. Liver Cu had a correlation coefficient of r = .39, .29 and .32 with total serum Cu, ceruloplasmin activity and TCA-soluble Cu, respectively.

The value of r = .37 between total serum Cu and ceruloplasmin activity for Cu criteria at the time of liver biopsy was found to be significantly ($P<.05$) different with the value of r = .62 between total serum Cu and ceruloplasmin activity at 3 days after biopsy (Table 9). A sample size of N = 74 was recorded for both cases.

## DISCUSSION

### Gross Clinical Condition

The steers in the experiment did not exhibit any clinical symptoms such as diarrhea, anorexia and loss of condition associated with consuming dietary molybdenum supplements. Apparently, the experimental treatments did not contain high enough Mo concentrations (A - 3.5 ppm, B - 7.5 ppm, C - 15.0 ppm and D - 50 ppm) to induce adverse effects. Huber et al. (1971) showed that cows fed a basal diet (6 ppm Cu, DM) containing 53 to 100 ppm Mo did not display any clinical signs of molybdenum toxicity. Also, the clinical signs of molybdenosis can be alleviated by high intakes of Cu (Ward 1978). All treatments in this study

Table 8. Experiment 1. Correlation coefficient among Cu criteria (3 days after biopsy) of beef steers[1-4]

| Y/X | Liver Cu | Serum Cu | Ceruloplasmin | TCA |
|---|---|---|---|---|
| Liver Cu | 1.00 | 0.39 | 0.29 | 0.32 |
| Serum Cu | - | 1.00 | 0.62 | 0.94 |
| Ceruloplasmin | - | - | 1.00 | 0.59 |
| TCA | - | - | - | 1.00 |

[1]N = 74

[2]X = independent variable and Y = dependent variable.

[3]Serum Cu (sampled 3 days after liver biopsy).

[4]Ceruloplasmin and TCA (TCA-soluble Cu) from serum samples 3 days after biopsy.

Table 9. Experiment 1. A test of significance of difference between two correlation coefficients

| Method[1] | Sample size (N) | $r^2$ | $Z^3$ | 1/(N-3) | D/S | Conclusion[4] |
|---|---|---|---|---|---|---|
| A | 74 | .37 | .388 | .014 | | |
| | | | | | 2.01 | * |
| B | 74 | .62 | .725 | .014 | | |
| | | | D=.337 | S=.028 | | |

[1]A = at time of liver biopsy.
B = 3 days after biopsy.

[2]Coefficient correlations between total serum Cu and ceruloplasmin activity.

[3]D = absolute difference.
S - SUM.

[4]N.S. = not significant (P>.05).
* = significant (P<.05).

contained concentrations of Cu and Mo in a 2:1 ratio.

One animal in group died following the third biopsy session, apparently from severe internal bleeding from the liver. Later in the same period, two more animals (each from groups A and B) developed chronic bloat and were shipped to the abattoir. There was no evidence that linked this condition specifically to the experimental treatments.

The remaining animals continued to gain weight throughout the experiment. Each animal displayed a good overall body condition and general lively disposition.

Copper Status - Liver Cu

At least one animal (regardless of breed) out of every treatment could be classified as marginally deficient in terms of liver Cu at the beginning of the experiment (Puls 1981). The liver copper content in the other animals was classified as copper adequate. The mean hepatic concentration for the treatment groups fell into the adequate copper classification. Throughout the experiment all treatment groups tended to accumulate hepatic copper (Figure 6). The degree to which copper was accumulated in the liver reflected the amount of copper supplemented in the diet (Table 6). Animals consuming the diet containing the highest copper concentration (group D) accumulated more than twice the copper in the liver and at a faster rate than the other three groups. Likewise, animals on the first diet (group A) had a very small elevation in hepatic Cu by the end of the experiment, while the animals on the second and third diets (groups B and C) accumulated copper moderately. All animals, regardless of group were classified as copper adequate in terms of

hepatic Cu by the end of the experiment.

Each diet contained dietary concentrations of Cu and Mo in a 2:1 ratio (Mason et al. 1971). The inability of dietary molybdenum to induce any adverse effects to liver Cu levels among treatment groups could be attributable to the formation of Cu:Mo complexes as theorized by Dowdy and Matrone (1968a, b). The dietary copper and molybdenum could have formed a biologically unavailable complex in which dietary copper combines with molybdenum in a molar ratio of 4:3 in the gut or bloodstream. Since a dietary Cu/Mo ratio of 2:1 is equal to a molar ratio of 9:3, any excess copper (after meeting Cu requirements) would remain unbound in the gut and blood of the animal. At higher dietary copper concentrations, the amount of copper unbound by Mo would be more than at a lower dietary copper concentration even though both diets contained Cu and Mo in the same 2:1 ratio. The pattern of storage of surplus amounts of copper in the liver by the different treatment groups is illustrated in Fig. 6.

Copper Status - Serum Cu Parameters

At the beginning of the experiment, one or more steers were marginally deficient with respect to total serum Cu in groups A, B and C (Puls 1981). In group D (highest Cu and Mo supplement), all four cattle were classified as adequate in total serum Cu. Serum Cu levels were not significantly ($P>.05$) affected by dietary treatment, however serum Cu levels were significantly ($P<.05$) affected by period effects. Although serum Cu levels of groups A, B and C neither decreased or increased outside of a range of ±10 μg/100 ml serum throughout the experiment,

group D animals had larger periodic changes (Fig. 7). In the first period, the serum Cu levels of group D fell 15 µg/100 ml serum but later rebounded approximately +30 µg/100 ml in periods II and III. Regardless of this behavior period x treatment interactions were found to be non-significant ($P>.05$).

Ceruloplasmin activities were not significantly ($P>.05$) affected by dietary treatment; however, the overall change in ceruloplasmin activities among cattle groups reflected the amount of copper supplemented in the diet. The magnitude of the difference between final and initial ceruloplasmin activities was the greatest in groups consuming diets of higher supplemental copper, namely groups C and D (Table 6). Regardless of treatment, serum ceruloplasmin activities were depressed during the first two periods of the experiment (Fig. 8). Spectrophotometric absorbance at 540 µm which was used to measure ceruloplasmin activity dropped about 20-30% among treatment groups by the beginning of the third period. From the third period and to the end of the experiment, ceruloplasmin activities rose in all four groups.

The correlation coefficient between total serum Cu and ceruloplasmin activity was $r = .37$ at the time of biopsy and $r = .62$, 3 days after biopsy. A significant difference ($P<.05$) was found between these two correlation coefficients (Table 9). This significant difference could be attributed to possible extreme observations of ceruloplasmin activity serum Cu measured at the time of liver biopsy. Regardless, only about one-seventh to one-third of the difference in ceruloplasmin levels were related to differences in total copper concentration in serum ($r^2 \times 100$, $r = .37, .63$) (Snedecor and Cochran 1980).

The relatively stable levels of total serum Cu (compared to ceruloplasmin activity) could be due to the formation of Cu-Mo complexes as outlined by Clawson et al. (1972). They stated that higher serum copper levels of animals receiving molybdenum most likely represented the accumulation of the copper-molybdenum complex in the blood.

The initial depression of ceruloplasmin activity and stable serum Cu levels could also be explained by the thiomolybdate theory. The molybdenum provided in the diets could combine with macronutrient levels of ruminal sulphide to yield thiomolybdate compounds. Excess of thiomolybdates are possibly absorbed in the digestive tract and subsequently combine with serum copper and plasma proteins to produce stable complexes (Mills et al. 1979). This bound copper becomes biologically unavailable for ceruloplasmin synthesis but still remains in the serum.

The later rebound in ceruloplasmin activity in all four treatment groups (Fig. 8) as well as higher overall changes in ceruloplasmin activity in treatment groups C and D was possibly because of buildup of excessive amounts of absorbed dietary copper that were not bound by molybdenum or thiomolybdates. Such additional copper causes an elevation in biologically available Cu and consequent stimulation of ceruloplasmin synthesis in the liver (Linder et al. 1979).

The proposed explanation seems to be contradicted by the stable levels of TCA-soluble Cu which is mostly comprised of ceruloplasmin and albumin Cu (Fig. 9). Furthermore, the correlation coefficient between TCA-soluble Cu and total serum Cu levels was $r = .94$. One may speculate that molybdenum/thiomolybdate may inactivate the ceruloplasmin molecule

in the blood without affecting the nature of its TCA-solubility. In addition, ceruloplasmin activity had a correlation coefficient with TCA-soluble Cu similar to that for ceruloplasmin activity and total serum Cu. Such a correlation of r = .59 shows that about one-third ($r^2$ x 100) of the difference in ceruloplasmin activity can be explained by changes in TCA-soluble copper levels.

Indicators of Cu Status

Throughout this experiment, several criteria were utilized in order to assess the effects of dietary treatment upon copper status in experimental yearlings. Since none of the animals was initially classified as Cu deficient (i.e. liver and serum Cu), gross clinical symptoms associated with a copper deficiency were neither observed nor expected during the experiment. Therefore, the choice of a suitable indicator of copper status was based primarily upon changes in the level of copper criteria which reflect changes in copper concentration in the animals body induced by the various Cu:Mo dietary treatments. The effect of period or time on treatment upon the level of copper criteria is also considered.

Of all the criteria used, liver Cu best reflected the influence of such dietary treatments and the duration of time in which they were fed to the cattle. The high degree of variability associated with different biopsy sites was minimized by taking the liver biopsy sample at the same point (between the 11th intercostal space) in each biopsy session. This point was marked by a small white scar which was easily observed one week after the first biopsy session.

On the other hand, serum copper levels unlike liver Cu did not reflect the effects of the Cu:Mo diets upon the copper state of the cattle. All serum samples reported were above 0.60 µg/ml and were found to be poorly correlated with liver Cu levels ($r = +.37$). Such results are in agreement with the findings of Claypool et al. (1975). They demonstrated that liver Cu and plasma (also serum) were poorly correlated when plasma copper levels were above 0.50 µg/ml and thus judged plasma Cu level as a poor indicator of copper status in cattle.

Although changes in ceruloplasmin activity among treatment groups were found to be non-significant and generally inconsistent with dietary levels of Cu and Mo, the overall changes in ceruloplasmin activity reflected the influence of the Cu:Mo diets. Ceruloplasmin analysis of serum has been accepted in many studies, as an indicator of Cu status in ruminants, yet results from one study to another are usually not comparable since ceruloplasmin activity is commonly reported in terms of arbitrary units. Standardization of bovine ceruloplasmin in the present experiment showed that the multiplication factor used to convert raw ceruloplasmin data into IEU was less than demonstrated by Rice (1962) using human serum (307.9 vs 349.0, respectively). This difference could be attributed to a species difference or to the questionable purity of the synthesized Bandowski's base crystals.

Like serum Cu, TCA-soluble Cu (both of which are highly correlated did not reflect the effect of Cu:Mo dietary treatments upon copper status of beef steers. The level of TCA-soluble Cu in total serum Cu generally remained at high levels throughout the experiment, showing little devi-

ation from initial values (with the exception of group D). Like total serum Cu and ceruloplasmin criteria, TCA-soluble Cu was also not significantly ($P>.05$) affected by dietary treatment.

## CONCLUSIONS

1. Steers did not differ significantly ($P>.05$) among treatment groups in live weight gain, ADG and dressed carcass weight.
2. Liver Cu levels of steers was significantly ($P<.05$) affected by treatment whereas serum Cu parameters did not differ appreciably ($P>.05$) among treatments.
3. Diets with higher concentrations of copper and molybdenum in a 2:1 ratio tended to cause increased liver Cu, serum Cu and serum ceruloplasmin activity levels more than treatments containing a marginal level of copper and molybdenum in the same 2:1 ratio.
4. Copper accumulated ($P<.01$) throughout the experiment in the liver. Although relatively stable, serum copper levels tended to increase with time on treatment. Ceruloplasmin activity just decreased and then increased through the experiment ($P<.01$) with small gains observed by the end of the experiment compared with initial values.
5. Liver Cu was judged as the best monitor to examine the effects of treatment diets on copper status of beef yearling steers on the basis that changes in liver Cu were the only parameter which appeared to be consistent with dietary Cu levels. Ceruloplasmin activity appears as an adequate indicator of copper status if based solely on overall results. However, like serum Cu and

TCA-soluble Cu, ceruloplasmin activity among the treatment groups was largely inconsistent with dietary levels of Cu and Mo.

6. Arbitrary enzyme units were chosen over IEU to express ceruloplasmin assay data due to analytical setbacks and inconsistencies of standardization procedures.

Experiment 2. The effect of different levels of sulphur in the diet on copper status and performance of beef yearling steers in a balanced 4x4 latin square.

## EXPERIMENTAL OBJECTIVES

1. To serve as a preliminary experiment for a seond study on the effects of dietary sulphur on yearling beef steers.
2. To study the effects of high levels of sulphur in the diet of yearling beef steers upon:
   a. feed palatability and intake.
   b. liver copper and serum copper parameters; total copper levels, ceruloplasmin activities (a Cu-dependent enzyme) and TCA-soluble copper fractions.
3. To determine the value of different copper criteria for assessing copper status in steers fed diets containing various levels of sulphur.

## MATERIALS AND METHODS

### Animals and Management

Four Selkirk-red yearlings weighing approximately 280 kg each were used in a balanced 4x4 latin square design.

The steers were housed in one pen which had both covered and open areas. The pen contained four feed boxes inside the barn and an automatic waterer outside the barn. Wood shavings were provided in the covered part of the pen whereas straw bedding was distributed on the

concrete in the outside area.

The steers were individually fed the respective diets by tying up the steers in the morning and giving the feed allowance. The steers were later released to allow watering for about one hour and retied to permit further feed consumption.

Design and Diets

One of four treatments was fed to each steer in a balanced Latin Square Design for four test periods of 28 days each. Between each test period, there was a 14 day rest period during which all animals received a resting diet (Table 10). The rest period represented an attempt to reduce residual effects from one treatment period to the next.

Each animal received 9 kg/day of the diets which were formulated to support an average daily gain of 0.9 kg/day by the heaviest steers (Table 11). The diet fed in pelleted form, consisted of alfalfa-brome-grass hay and barley mixture to which various levels of sodium sulphate were added in the premix to establish the various sulphur treatments. The four treatments containing sulphur on an actual basis were: (A) 0.2% S, (B) 0.3% S, (C) 0.4% S and (C) 0.5% S. The resting diet fed to the cattle was the 0.2% S treatment. The chemical compositions of the diets are listed in Table 12.

Sampling Procedure

Feed consumption of each animal was obtained daily by feeding 9 kg of pellets in the morning and weighing remaining amounts in the afternoon.

Table 10. Experiment 2. Balanced 4x4 latin square with rest periods[1]

| | | Animal | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| | I | A | B | C | D |
| P | rP | r | r | r | r |
| E | | | | | |
| R | II | B | D | A | C |
| I | rP | r | r | r | r |
| O | | | | | |
| D | III | C | A | D | B |
| | rP | r | r | r | r |
| | IV | D | C | B | A |

[1]rP = rest period

Treatment - (A) - 0.2% S
(B) - 0.3% S
(C) - 0.4% S
(D) - 0.5% S
(r) - resting ration

Table 11. Experiment 2. Composition of basal and treatment diets fed to beef yearling steers in a 4x4 latin square

| Composition | Basal diet |
|---|---|
| Alfalfa-bromegrass hay (%) | 52.5 |
| Barley (%) | 45.0 |
| Premix (%) | 2.5 |
| Urea (%)[ab] | 0.5 |
| NaCl (%)[ac] | 0.5 |
| ZnO (mg/kg)[ad] | 25.0 |
| CuO (mg/kg)[a] | 17.3 |
| $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ | 5.5 |

| | Treatments | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | R |
| $Na_2SO_4$ (%)[e] | 0.00 | 0.47 | 0.90 | 1.36 | 0.00 |

[a]Ingredients listed under premix (including $Na_2SO_4$) are supplemented in the premix of the diet. The amount of these ingredients are recorded as the level in the complete diet.

[b]Urea = 45% N.

[c]Cobalt-iodized salt.

[d]ZnO = 80.3% Zn.

[e]R = resting ration.

Table 12. Experiment 2. Analyzed chemical composition (dry matter basis) of diets fed to beef steers

| Diet[a] | |
|---|---|
| Dry matter (%) | 92.4 |
| Nitrogen (%) | 2.29 |
| Protein (Nx6.25) (%) | 14.30 |
| ADF (%) | 20.2 |
| Energy (kJ/g) | 16.8 |
| Ca (%) | 0.60 |
| P (%) | 0.34 |
| Mg (%) | 0.21 |
| Zn (mg/kg) | 38.7 |
| Fe (mg/kg) | 118.2 |
| Mn (mg/kg) | 18.2 |
| Cu (mg/kg) | 18.9 |
| Mo (mg/kg) | 6.0 |
| S (g/kg) | |
| Treatment[b] | |
| A | 2.1 |
| B | 2.7 |
| C | 3.8 |
| D | 4.4 |
| r[b] | 2.2 |

[a]Representative samples of all 4 treatments and resting diet was obtained and thoroughly mixed together for analysis for protein, ADF, energy content, Ca, P and listed microminerals.

[b]r = resting diet.

All animals were weighed and liver biopsies taken at the end of each test period, thus liver samples were obtained approximately every 6 weeks. Blood samples via jugular vein were obtained weekly throughout the experiment. Liver biopsy and blood sampling techniques were the same as for Experiment 1.

Liver and serum samples (only taken at the time of biopsy) were analyzed for copper concentration by methods described in Experiment 1. Ceruloplasmin activity and TCA-soluble copper levels in serum were also determined by procedures outlined in Experiment 1. Similarly, the analytical methods used for protein, energy, ADF and macro- and micro-mineral content of feeds were as in the previous experiment.

### Statistical Analysis

A 4x4 balanced latin square analysis was used to estimate the significance ($P<.05$, $P<.01$) of the effects of treatment, period and animal on feed consumption of treatment diets and on changes of liver Cu, and serum criteria from initial values (symbolized as $\Delta_1$). Each period consisted of 4 weeks on the test diet followed by 2 weeks on the resting diet.

Correlation coefficients between liver Cu, serum Cu, ceruloplasmin activity and TCA-soluble Cu levels were determined by simple linear regression.

## RESULTS

There was a mechanical problem with the weigh scale for much of the

experiment so only results for live weight gain and average daily gain (ADG, kg/day) based on 168 days (length of experiment) are reported (Table 13). The ADG for steers in the 168 day experiment were as follows: #56 - 1.07, #86 - 1.15, #79 - 1.08 and #93 - 1.18 kg/day, respectively.

Differences in feed consumption among steers on dietary treatments were not significant ($P>.05$) and ranged from 6.29-8.28 kg/day (Table 14). Intake differed significantly ($P<.01$) among periods with consumption being the lowest in period one.

Liver Cu levels were not significantly ($P>.05$) affected by treatment. The only decrease in liver Cu in the experiment was recorded for animals consuming dietary treatments C (0.4% S) and D (0.5% S) in period one (Tables 22A and 15) where there was a loss of -1.95 ppm and -4.95 ppm of hepatic copper respectively. Liver Cu levels were, however, significantly different among steers ($P<.05$) and period ($P<.01$) (Table 22A). All animals had accumulated substantial amounts of hepatic Cu (37.5-68.8 ppm) by the end of the experiment (Table 15).

Serum Cu levels were not significantly ($P>.05$) different among steer treatments or periods (Table 23A). For most of the experiment serum Cu levels in the steers remained within a narrow range (0.52-0.70 µg/ml) (Table 16).

Ceruloplasmin activities were not significantly different ($P>.05$) among dietary treatments but were significantly ($P<.01$) different among steers and periods (Table 24A). All four animals had increased ceruloplasmin activities by the end of the experiment (Table 17).

TCA-soluble Cu levels (µg/ml in serum, or as a % of serum Cu) were

Table 13. Experiment 2. Body weight gain (kg)[1,2]

| Animal | Initial weight (kg) | Final weight (kg) | Δ | ADG |
|---|---|---|---|---|
| 56 | 287 | 467 | 180 | 1.07 |
| 86 | 275 | 468 | 193 | 1.15 |
| 79 | 265 | 446 | 181 | 1.08 |
| 93 | 293 | 491 | 198 | 1.18 |

[1]Δ = Live weight gain (kg).

[2]ADG = Average daily gain (kg/day), 168 day basis.

Table 14. Experiment 2. Feed consumption (kg/day)

| Period | Animal 56 | 86 | 79 | 93 |
|---|---|---|---|---|
| I | 7.05(A) | 6.26(B) | 6.93(C) | 6.87(D) |
| II | 7.84(B) | 7.22(D) | 8.51(A) | 8.01(C) |
| III | 8.32(C) | 7.85(A) | 7.68(D) | 8.71(B) |
| IV | 7.88(D) | 7.93(C) | 7.84(B) | 8.28(A) |

Treatment - (A) - 0.2% S

(B) - 0.3% S

(C) - 0.4% S

(D) - 0.5% S

Table 15. Experiment 2. Liver Cu (mg/kg, wet basis)

| | Animal | | | |
|---|---|---|---|---|
| | 56 | 86 | 79 | 93 |
| Initial values | 15.28 | 25.82 | 35.58 | 31.44 |
| Period I | (A) 31.46 | (B) 30.67 | (C) 34.34 | (D) 26.49 |
| | (16.18) | ( 4.82) | (-1.24) | (-4.95) |
| II | (B) 52.36 | (D) 33.94 | (A) 46.09 | (C) 49.76 |
| | (37.08) | ( 8.12) | (10.51) | (18.32) |
| III | (C) 61.68 | (A) 46.72 | (D) 64.53 | (B) 74.56 |
| | (46.40) | (20.90) | (28.95) | (43.14) |
| IV | (D) 72.26 | (C) 63.32 | (B) 83.60 | (A) 100.24 |
| | (56.98) | (37.50) | (48.02) | (68.80) |

Treatment - (A) - 0.2% S
(B) - 0.3% S
(C) - 0.4% S
(D) - 0.5% S

(*) - numbersin brackets represent Δ liver Cu (mg/kg, wet basis) from initial values ($\Delta_1$).

Table 16. Experiment 2. Serum Cu (µg/ml)

| | | Animal | | | |
|---|---|---|---|---|---|
| | | 56 | 86 | 79 | 93 |
| Initial values | | 0.62 | 0.64 | 0.62 | 0.62 |
| Period | I | (A) 0.90 (0.28) | (B) 0.75 (0.11) | (C) 0.65 (0.03) | (D) 0.58 (-0.04) |
| | II | (B) 0.66 (0.04) | (D) 0.71 (0.07) | (A) 0.58 (-0.04) | (C) 0.68 (0.06) |
| | III | (C) 0.53 (-0.09) | (A) 0.60 (-0.04) | (D) 0.54 (-0.08) | (B) 0.52 (-0.10) |
| | IV | (D) 0.64 (0.02) | (C) 0.62 (-0.02) | (B) 0.70 (0.08) | (A) 0.69 (0.07) |

Treatment - (A) - 0.2% S
(B) - 0.3% S
(C) - 0.4% S
(D) - 0.5% S

(*) - Numbers in brackets represent Δ serum Cu (µg/ml) from initial values ($\Delta_1$).

Table 17. Experiment 2. Ceruloplasmin activity (abs at 540 mμ)

| | Animal | | | |
|---|---|---|---|---|
| | 56 | 86 | 79 | 93 |
| Initial values | 0.083 | 0.025 | 0.010 | 0.008 |
| Period I | (A) 0.103 | (B) 0.102 | (C) 0.080 | (D) 0.105 |
| | (0.020) | (0.077) | (0.070) | (0.097) |
| II | (B) 0.064 | (D) 0.015 | (A) 0.037 | (C) 0.047 |
| | (-0.019) | (-0.010) | (0.027) | (0.039) |
| III | (C) 0.084 | (A) 0.088 | (D) 0.052 | (B) 0.085 |
| | (0.001) | (0.063) | (0.042) | (0.077) |
| IV | (D) 0.114 | (C) 0.100 | (B) 0.090 | (A) 0.129 |
| | (0.031) | (0.075) | (0.080) | (0.121) |

Treatment - (A) - 0.2% S
(B) - 0.3% S
(C) - 0.4% S
(D) - 0.5% S

(*) - numbers in brackets represent Δ ceruloplasmin activity (abs. at 540 mμ) from initial values ($\Delta_1$)

not significantly (P>.05) affected among steers, treatments or periods (Table 25A, 26A). Compared to initial values there was a tendency (except steer #93) for a large increase in the % of TCA-soluble Cu at the end of the first period and then there was a subsequent drop by the second or third period (Table 18 and 19). All animals, however, ended the experiment with a substantial increase in the percentage of TCA-soluble Cu in total serum Cu. Although diets were found not to significantly (P>.05) affect the level of TCA-soluble Cu in the serum Cu, all animals exhibited a TCA-soluble Cu below 90.0% after consuming treatment D (0.5% S) for one month while TCA-soluble Cu was close to 100% total serum Cu in animals consuming treatment A (0.2% S).

The relationships among copper criteria were examined using correlation analysis, as shown in Table 20. The associations between liver and blood copper parameters range from r = -.9 with total serum Cu to r = -.30 with % TCA-soluble Cu. The dimensions of correlation coefficients among serum parameters were variable (Table 12). Serum Cu had a correlation coefficient of -0.04 with ceruloplasmin activity but a moderately high association with TCA-soluble Cu (r = 0.62). TCA-soluble Cu which is supposedly mainly comprised of ceruloplasmin was shown to have almost no association (r = 0.02) with ceruloplasmin activity levels.

## DISCUSSION

### Performance - Feed Intake

There were no depressions in feed intake associated with feeding high levels of sulphur in the diet. Similarly, Pendlum et al. (1976)

Table 18. Experiment 2. TCA-soluble Cu (µg/ml)

| | | Animal | | | |
|---|---|---|---|---|---|
| | | 56 | 86 | 79 | 93 |
| Initial values | | 0.48 | 0.54 | 0.40 | 0.55 |
| Period | I | (A) 0.88 (0.40) | (B) 0.79 (0.25) | (C) 0.70 (0.30) | (D) 0.51 (-0.04) |
| | II | (B) 0.64 (0.16) | (D) 0.63 (0.09) | (A) 0.55 (0.15) | (C) 0.64 (0.09) |
| | III | (C) 0.45 (-0.03) | (A) 0.58 (0.04) | (D) 0.47 (0.07) | (B) 0.48 (-0.07) |
| | IV | (D) 0.58 (0.10) | (C) 0.56 (0.02) | (B) 0.63 (0.23) | (A) 0.69 (0.14) |

Treatment - (A) - 0.2% S
(B) - 0.3% S
(C) - 0.4% S
(D) - 0.5% S

(*) - Numbers in brackets represent Δ TCA-soluble Cu (µg/ml) from initial values ($\Delta_1$).

Table 19. Experiment 2. TCA-soluble Cu (% of serum Cu)

| | Animal | | | |
|---|---|---|---|---|
| | 56 | 86 | 79 | 93 |
| Initial values | 76.60 | 84.47 | 77.67 | 88.22 |
| Period I | (A) 97.56 | (B) 105.21 | (C) 107.98 | (D) 87.92 |
| | (20.96) | (20.74) | (30.31) | (-0.30) |
| II | (B) 97.42 | (D) 88.42 | (A) 95.47 | (C) 93.27 |
| | (20.82) | ( 3.95) | (17.80) | ( 5.05) |
| III | (C) 84.40 | (A) 96.67 | (D) 87.69 | (B) 92.18 |
| | ( 7.80) | (12.20) | (10.02) | ( 3.96) |
| IV | (D) 89.84 | (C) 90.80 | (B) 88.92 | (A) 99.57 |
| | (13.24) | ( 6.33) | (11.25) | (11.35) |

Treatment - (A) - 0.2% S
(B) - 0.3% S
(C) - 0.4% S
(D) - 0.5% S

(*) - numbers in brackets represent Δ TCA-soluble (% of serum Cu) initial values ($\Delta_1$).

Table 20. Experiment 2.[1-3] Correlation coefficients among Cu criteria of beef steers

| Y/X | Liver Cu | Serum Cu | Ceruloplasmin | TCA | PR |
|---|---|---|---|---|---|
| Liver Cu | 1.00 | -0.19 | 0.09 | -0.03 | -0.30 |
| Serum Cu | - | 1.00 | -0.04 | 0.62 | 0.45 |
| Ceruloplasmin | - | - | 1.00 | -0.02 | 0.25 |
| TCA | - | - | - | 1.00 | 0.29 |
| PR | - | - | - | - | 1.00 |

[1] N = 16.

[2] X = Independent variable and Y = dependent variable.

[3] Liver Cu (ppm), serum Cu (µg/ml), ceruloplasmin (abs. at 540 mµ), TCA = TCA-soluble Cu (µg/ml) and PR = soluble Cu as a % of serum Cu.

showed that diets containing 0.11%, 0.26% and 0.45% S did not affect feed intake, growth and feed efficiency. The composition of the diets in that study differed from those in the present experiment in that, Pendlum et al. (1976) included 2.0% molasses in the diet.

The animals frequently refused 1.0-2.5 kg/day of feed regardless of treatment. The failure to consume the entire daily feed allowance may be attributed to the tethering of the steers. The animals were forced to feed in a 3 hour morning and afternoon period and were relatively stationary in position (allowed to lay down) and were without water during the feeding periods. When the resting diet was fed between dietary treatment periods, the steers were not tethered and none of the feed was refused.

Copper Status - Liver Cu

According to the definition of Puls (1981), only one steer could be classified as marginally deficient with respect to liver Cu at the start of the experiment. The hepatic copper content of the other three steers was classified as Cu adequate. Copper tended to accumulate in the livers resulting in doubling or tripling the concentrations of hepatic copper. By the end of the experiment, all four animals were considered adequate in liver Cu.

The lack of response of liver Cu to dietary S treatments was possibly due to a flaw of sampling design as a result of the utilization of a balanced latin square design in combination with resting periods. Liver biopsy samples were obtained only at the end of each dietary period

rather than at both the beginning and end of the period. Therefore, changes in liver Cu were observed following the combined effects of the resting and dietary periods rather than for the dietary period alone. Also a dietary period of 28 days (see Materials and Methods) was possibly not long enough to elicit a hepatic copper response. Furthermore, the duration of the resting period was possibly too short to avoid the residual effects of one treatment upon another.

The differences in hepatic responses among steers were attributed to different physiological mechanisms of one animal compared to another. The hepatic retention of dietary copper may differ among the four pen mates. Liver Cu variations as a direct result of the liver biopsy technique were minimized by obtaining the liver sample at the 11th intercostal space of each biopsy period. The animals were not adversely affected by the biopsy procedure which was conducted every six weeks.

Copper Status - Serum Cu Parameters

All animals were classified as marginally deficient (Puls 1981) with respect to serum Cu levels and with the exception of one animal, small gains were measured by the end of the experiment. Overall serum Cu levels were relatively stable throughout the experiment, and there appeared to be no systematic changes in serum Cu in animals being switched from one diet to another.

The stability of serum Cu levels in animals consuming diets of high dietary copper (20 mg/kg) could be due to the presence of dietary molybdenum (5 mg/kg) and various sulphur levels incorporated in the treatment

diets. Copper could combine with elevated ruminal sulphide levels (caused by added sodium sulphate) to form an insoluble CuS complex as outlined by Suttle (1974b). Enough excess unbound copper could be absorbed to maintain serum Cu levels or even to slightly elevate serum Cu levels. The dietary molybdate might also combine with sulphide (regardless of treatment) to form thiomolybdates in the rumen. These thiomolybdate compounds might bind copper in the gut and thus cause a reduction in Cu absorption.

The apparent lack of systemic effects of thiomolybdates, as shown by elevated ceruloplasmin activity and high percentage of TCA-soluble Cu in the serum, was possibly because of a shortage of absorbable $MoS_4^{-2}$ with an affinity for Cu (Suttle and Field 1983). However, there was a tendency for TCA-soluble Cu (%) to be lower when animals consumed the highest sulphur diet (0.5%), therefore, there were possibly some systemic effects.

Indicators of Copper Status

Liver Cu, serum Cu, ceruloplasmin activity and TCA-soluble Cu were employed to evaluate the copper status in yearling steers consuming diets of various sulphur concentrations. Since these steers were initially classifiable as marginally deficient or adequate in copper with respect to both liver and serum Cu, changes in copper status were evaluated on the basis of alterations of the measured criteria by the dietary treatments.

Liver Cu was judged as an unsatisfactory monitor of the effects of dietary treatments upon copper status of beef steers. The validity of

using liver Cu as a measurement of copper status in the present experiment was questioned because of an error made in the design of the sampling procedure. Hepatic Cu levels also reflected the accumulation of copper supplemented in the basal diet rather than the effects of the sulphur treatments. Serum Cu and ceruloplasmin levels also showed no consistent pattern which reflected the effects of sulphur upon copper status of beef steers.

Of the four criterion, TCA-soluble Cu was judged as a fair copper indicator to record the effects of sulphur treatments on copper status of beef steers. TCA-soluble Cu levels were generally lower in animals consuming the diets of higher sulphur content compared to the TCA-soluble Cu levels of the same animals consuming diets of low sulphur content. Like the other criterion, all four animals did have an increase in the percentage of TCA-soluble Cu in the total serum by the end of the experiment. This accumulation of TCA-soluble Cu reflects the supplemental copper in the basal diet. Despite these changes in TCA-soluble Cu levels, the choice of an adequate copper indicator in this study was not apparent largely due to the inconclusive information generated by the other copper criteria.

## CONCLUSIONS

1. Feed consumption was not significantly ($P>.05$) affected by dietary treatment. On a short term basis, all cattle accepted the dietary treatments containing various levels of sulphur.
2. Liver Cu and serum Cu parameters were not significantly ($P>.05$) affected by dietary sulphur treatment.

3. Copper accumulated ($P<.01$) in the liver throughout the experiment. Ceruloplasmin activity levels were significantly ($P<.01$) elevated by the end of the experiment. Serum Cu and TCA-soluble Cu levels were not significantly ($P>.05$) altered by time on treatment. Serum Cu levels were relatively steady throughout the duration of the experiment.
4. Liver Cu ($P<.05$) and ceruloplasmin ($P<.01$) levels were significantly different among steers, whereas serum Cu and TCA-soluble Cu levels were not ($P>.05$).
5. The choice of an adequate indicator of copper status of beef steers remains inconclusive because of sampling errors made in the experiment.

Experiment 3. The effect of different levels of sulphur in the diet on copper status and performance of beef yearling steers fed for 118 days.

## EXPERIMENTAL OBJECTIVES

1. To study the effects of different levels of sulphur in the diet of beef cattle on:
   a. palatability and feed intake.
   b. the levels of liver copper, serum total copper, ceruloplasmin activity (a Cu-dependent enzyme) and TCA-soluble Cu.
   c. the accumulation or elimination of copper from the liver or serum.
   d. the redistribution of copper in the serum in beef cattle.
2. To determine the value of different copper criteria for assessing copper status in beef cattle fed diets with a high sulfur content.

## MATERIALS AND METHODS

### Animals and Management

Sixteen beef steers were used in a split-plot design with repeated measurements.

Chosen from a pool of 80 cattle on the basis of size, initial live weight and overall condition, the sixteen animals consisted of 2 Herefords, 3 Shorthorn-Hereford crossbreds and 11 Selkirk-red cattle. These cattle were allotted to four treatment pens (4 per pen) according

to weight and breed (Table 34A) and were housed in an open end pole barn. Each pen contained a feed bunk and an automatic waterer. Straw bedding was provided on the concrete floors of the pens.

At the beginning of the experiment, all animals were injected with Vitamin A, D and E preparation.

Design and Diet

One group of steers was assigned to each of four diet treatments for a period of approximately four months. The pelleted diets were similar in basal and treatment formulation (Table 21) to those used in Experiment 2, but 3.0% molasses was added to reduce possible unpalatability associated with feeding high levels of sulphur. The treatment groups contained sulphur on an actual basis as follows: (A) 0.2% S, (B) 0.3% S, (C) 0.4% S and (D) 0.5% S. Each group received a limit of 36 kg of diet per day. An adjustment period of 1 week was allowed for steers to adapt to the diets. The chemical composition of each treatment diet is shown in Table 22.

Sampling Procedure

Feed consumption for each pen was recorded daily. The animals were weighed and liver biopsies were taken approximately every 28 days. Jugular blood samples were taken on a weekly basis. As for Experiment 1, liver samples for the last period were obtained at the abbatoir. Penicillin injections were also given to each animal just before taking liver biopsies. Liver biopsy and blood sampling as well as sample

Table 21. Experiment 3. Composition of basal and treatment diets fed to beef yearling steers for 118 days

| Composition | Basal diet |
|---|---|
| Alfalfa-bromegrass hay (%) | 40.9 |
| Barley (%) | 53.2 |
| Molasses (%) | 3.0 |
| Premix (%)[a] | 2.5 |
| Urea (%)[b] | 0.5 |
| NaCl (%)[c] | 0.5 |
| ZnO (mg/kg)[d] | 10.4 |
| CuO (mg/kg) | 18.2 |
| $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (mg/kg) | 6.8 |

| | Treatments | | | |
|---|---|---|---|---|
| | A | B | C | D |
| $Na_2SO_4$ (%) | 0.10 | 0.50 | 1.00 | 1.50 |

[a]Ingredients listed under premix (including $Na_2SO_4$) are supplemented in premix of the diet. The amount of these ingredients are recorded as the level in the complete diet.

[b]Urea = 45% N.

[c]Cobalt-iodized salt.

[d]ZnO = 72.0% Zn.

Table 22. Experiment 3. Chemical composition (analyzed) of treatment diets (dry matter basis) fed to beef steers for 118 days

| | |
|---|---|
| Dry matter (%) | 91.0 |
| Nitrogen (%) | 2.25 |
| Protein (Nx6.25) (%) | 14.08 |
| ADF (%) | 15.4 |
| Energy (kJ/g) | 16.6 |
| Ca (%) | 0.60 |
| P (%) | 0.34 |
| Mg (%) | 0.21 |
| Zn (mg/kg) | 38.7 |
| Fe (mg/kg) | 118.2 |
| Mn (mg/kg) | 18.2 |
| Cu (mg/kg) | 18.9 |
| Mo (mg/kg) | 6.0 |
| S/kg | |
| Treatment | |
| 0.2% S | 1.7 |
| 0.3% S | 2.9 |
| 0.4% S | 3.8 |
| 0.5% S | 4.5 |

[a]Other than sulphur content, representative samples of all diets were obtained and thoroughly mixed together for analysis.

determinations were as detailed for Experiment 1.

Statistical Analysis

The significance ($P<.05$, $P<.01$) of the main effects of treatment, period and treatment x period interactions on changes in the level of copper in the liver and serum Cu parameters from time zero data were tested by split-plot analysis. Details for this statistical design are outlined as in Experiment 1.

Tukey's multiple range test was employed to determine significant differences ($P<.05$) among treatment groups with respect to mean overall changes (over duration of the experiment) in weight gain, liver Cu and serum Cu criteria. Furthermore, the Tukey's multiple range test was used to determine significant differences among treatment means of initial liver Cu levels. More detail for this statistical design is given in Experiment 1.

A randomized complete block design was used to test the significance ($P<.05$) of the main effects of treatment, and period on feed consumption of beef yearling steers.

Correlation coefficients between liver Cu and serum Cu and related parameters were determined by simple linear regression. A test of significance ($P<.05$) of the difference between two correlation coefficients was also utilized.

## RESULTS

Live weight gain, ADG and dressing percentages were not significantly different ($P>.05$) among dietary treatments (Table 23). The average daily

Table 23. Experiment 3. The effect of different levels of sulphur in the diet on weight gain ($\Delta_2$) in beef steers fed for 118 days[1]

| Treatment[2] | Live weight gain (kg) | ADG[3] | Dressed wt. (% of live wt.) |
|---|---|---|---|
| 0.2% | 99.0[a] | 0.84[a] | 52.43[a] |
| 0.3% S | 106.0[a] | 0.90[a] | 53.00[a] |
| 0.4% S | 91.5[a] | 0.78[a] | 53.68[a] |
| 0.5% S | 103.0[a] | 0.88[a] | 52.89[a] |

[1]Values within columns with different superscripts are significantly different ($P<.05$).

[2]Treatment groups are referred to in text as Groups A, B, C, D, respectively (in order of increasing sulphur concentration in treatment).

[3]ADG = Average daily gain (kg/day), 118 day basis.

gain among treatment groups ranged from .78-.90 kg/day. After slaughter, there was no significant (P>.05) difference among treatment groups in carcass yield (DCY). The DCY from all steers ranged from 50-55%. From the total group of 15 graded carcasses there were 10 A1, 4 A2 and one underfinished B1 carcass. Such grades were evenly distributed among treatment groups. One animal was sold privately and therefore not graded.

Feed consumption was not significantly (P>.05) influenced by dietary treatment of sulphur (Table 40A). Although 36 kg/day was fed to each pen the mean consumption among pens was A = 32.05, B = 33.35, C = 32.36 and D = 33.78 kg of feed/day (Table 24). On the other hand, feed consumption was significantly (P<.01) affected by period. Feed consumption by the steers improved in the latter periods compared to the first two periods.

Although hepatic copper levels markedly increased (P>.01) throughout the experiment (Fig. 10), the increases were not significantly different (P>.05) among treatments (Table 35A and 25). There was a tendency for animals on treatments A and D to accumulate liver copper at a faster rate and greater magnitude than steers on treatments B and C. There was no significant (P>.05) treatment x period interaction. Furthermore, animals on treatment B started the experiment with the lowest hepatic copper content but this level was not significantly different (P>.05) from other groups' initial hepatic copper values (Table 42A). The mean increases in liver Cu (ppm) for the treatment groups were: A = 79.30, B = 62.66, C = 50.57 and D = 84.14.

Table 24. Feed consumption (kg/4 animals) of beef steers fed different levels of sulphur in the diet for 118 days[1]

| | | Treatment | | | |
|---|---|---|---|---|---|
| | | 0.2% S | 0.3% S | 0.4% S | 0.5% S |
| Period | I | 850 | 864 | 864 | 864 |
| | II | 944 | 953 | 891 | 972 |
| | III | 1007 | 1113 | 1049 | 1077 |
| | IV | 981 | 1005 | 1014 | 1073 |
| | Total | 3782 | 3985 | 3818 | 3986 |
| | Ave. (kg/day) | 32.05 | 33.35 | 32.36 | 33.78 |

[1]Average feed consumption = 118 day basis.

Figure 10. Experiment 3. The effect of different levels of sulphur in the diet on LIVER Cu of beef steers fed for 118 days.

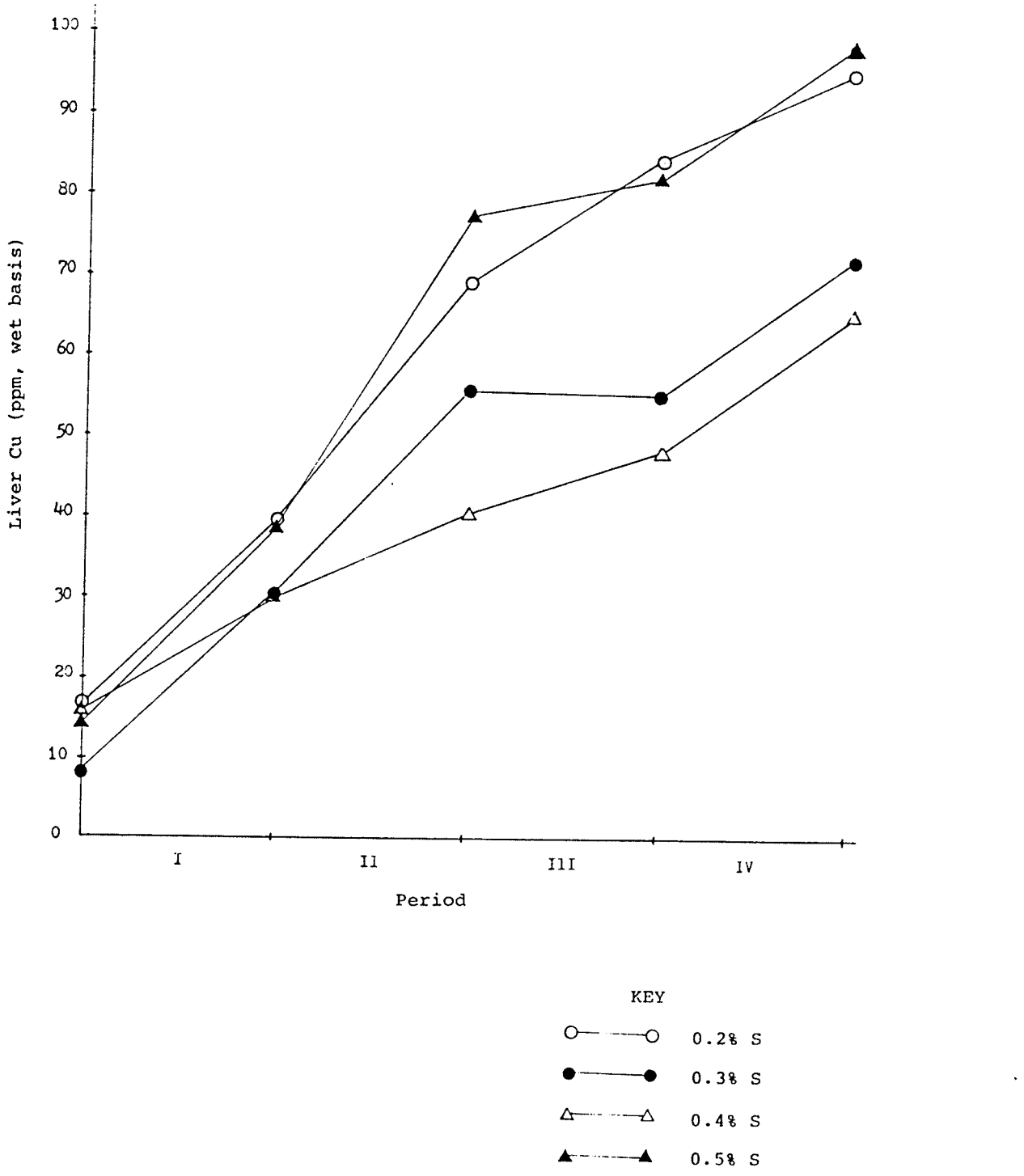


Table 25. Experiment 3. The effect of different concentrations of sulphur on changes ($\Delta_2$) in liver Cu, ceruloplasmin activity and TCA-soluble Cu of beef steers fed for 118 days[1]

| Treatment[2] | Liver Cu ($\Delta_2$)[3] | Serum Cu ($\Delta_2$)[4] | Ceruloplasmin activity ($\Delta_2$)[5] | TCA-soluble Cu ($\Delta_2$)[6] |
|---|---|---|---|---|
| 0.2% S | $79.30^{a}$ | $-0.023^{a}$ | $-0.011^{ab}$ | $-0.052^{a}$ |
| 0.3% S | $62.66^{a}$ | $0.040^{a}$ | $0.013^{a}$ | $0.014^{a}$ |
| 0.4% S | $50.57^{a}$ | $-0.054^{a}$ | $-0.033^{b}$ | $-0.104^{a}$ |
| 0.5% S | $84.14^{a}$ | $-0.071^{a}$ | $-0.027^{ab}$ | $-0.145^{a}$ |

[1]Values with different superscripts are significantly different ($P<.05$).

[2]Treatment groups are referred to in text as Groups A, B, C, D, respectively (in order of increasing sulphur concentrations in treatment).

[3]mg/kg of Cu in liver, wet basis.

[4]µg/ml of Cu in serum.

[5]Abs. at 540 mµ.

[6]µg/ml of Cu in TCA-soluble serum fraction.

By the end of the experiment serum Cu levels were not significantly different ($P>.05$) from initial values (Table 36A and 25) but levels were changed ($P<.01$) in the intervening period samples (Fig. 11). Dietary treatments did not significantly ($P>.05$) alter serum Cu levels among treatment groups (Table 36A). However, animals on treatment B starting with the lowest serum Cu levels compared to the other three groups, finished the experiment with the only positive change (not significant at $P>.05$, Table 44A) in serum Cu. The other three groups had slight overall serum Cu decreases, with a tendency for steers given the diets of high sulphur content (namely C - 0.4% and D - 0.5% S) to have a larger overall decline in serum Cu than steers consuming the diet with the lowest sulphur content (A - 0.2% S).

Serum ceruloplasmin activity was significantly ($P<.05$) affected by dietary treatment (Tables 37A, 25 and Fig. 12) as well as by period ($P<.05$). Treatment x period interactions were not significant ($P>.05$). The animals in group B had the lowest initial ceruloplasmin activity level of all four groups. However, the animals in group B had increased mean ceruloplasmin activity levels at the end of the experiment. In contrast, the other three groups had decreased overall ceruloplasmin activity with groups C and D having the greatest losses respectively.

Over the duration of the experiment, the TCA-soluble Cu levels (absolute or as a percentage of total serum Cu) were not significantly ($P>.05$) affected by dietary treatment (Tables 38A, 39A and 25, Fig. 13), but levels changed ($P<.01$) with time on treatment. Steers on treatment B had the lowest initial absolute TCA-soluble Cu level of all four groups.

Figure 11. Experiment 3. The effect of different levels of sulphur in the diet on SERUM Cu of beef steers fed for 118 days.

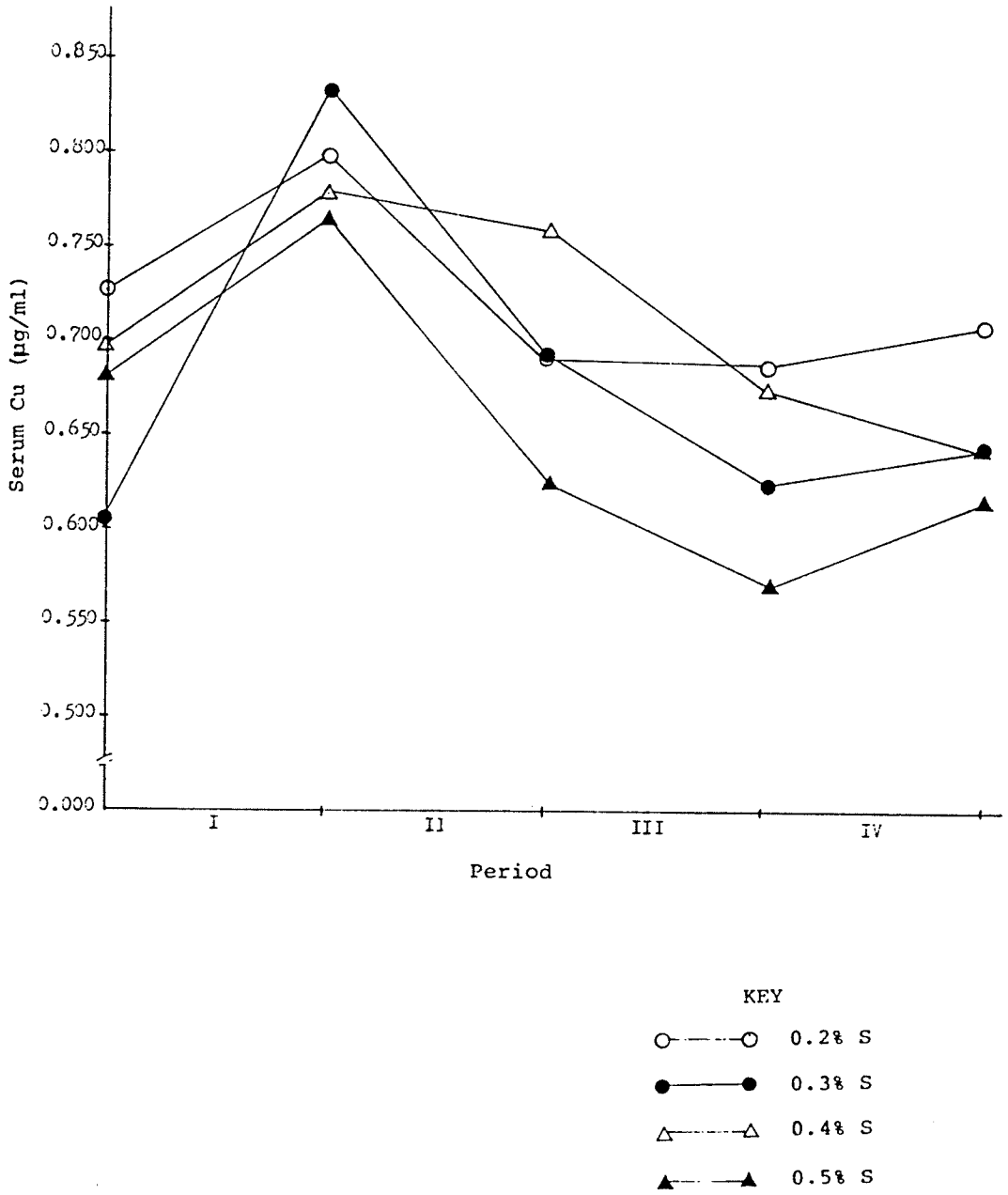


Figure 12. Experiment 3. The effect of different levels of sulphur in the diet on CERULOPLASMIN ACTIVITY of beef steers fed for 118 days.

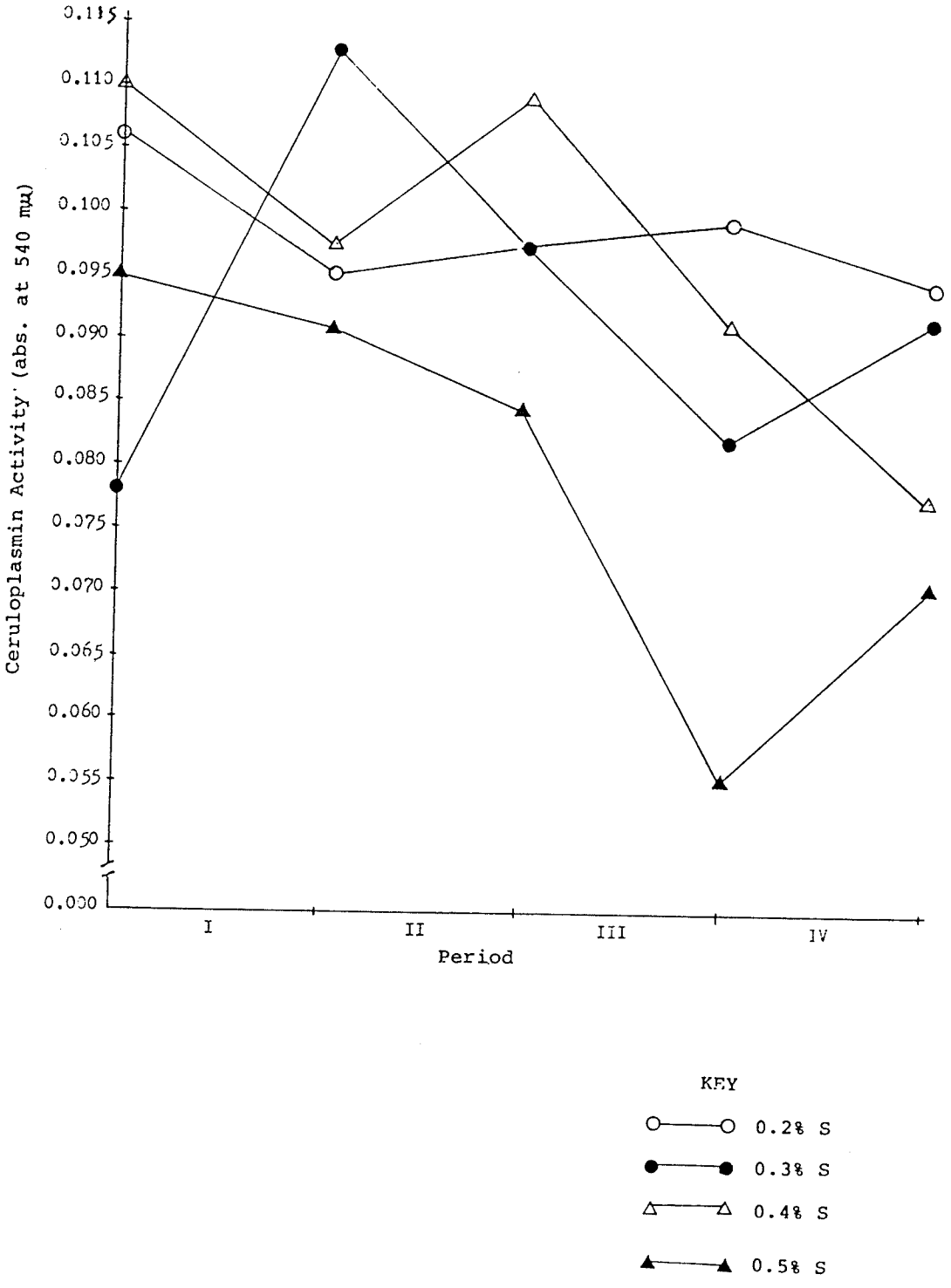
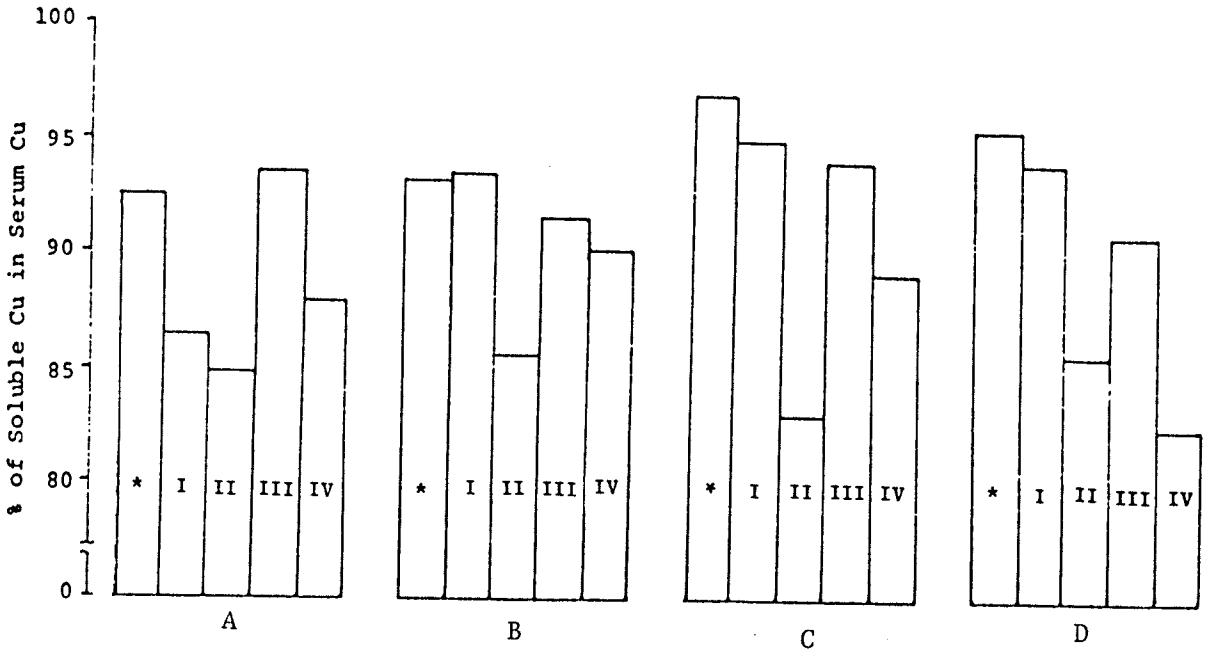


Figure 13. Experiment 3. The effect of different levels of sulphur in the diet on the % of TCA-SOLUBLE Cu of beef steers fed for 118 days.




KEY

A = 0.2% S
B = 0.3% S
C = 0.4% S
D = 0.5% S

* = initial period
I = period I
II = period II
III = period III
IV = period IV

Group A had the lowest initial percentage of TCA-soluble Cu in total serum Cu; 92.47% versus 93.14%, 96.97% and 95.35% in groups B, C and D respectively. By the end of the experiment, steers in group B had a small decline in TCA-soluble Cu (3.00%) while the means for groups A, C and D decreased by 4.68, 7.88 and 12.95% respectively, compared with initial values. TCA-soluble Cu made up 83 to 97% of mean total serum copper (Fig. 13).

Correlation coefficients showing the relationships between liver and serum Cu parameters are listed in Table 26. The correlation coefficients with liver Cu ranged from $r = -0.18$ for total serum Cu to $r = -0.31$ with TCA-soluble Cu levels. Total serum Cu had high correlation coefficients with ceruloplasmin ($r = 0.75$) and TCA-soluble Cu ($r = 0.84$) which were not significantly different ($P>.05$) with one another (Table 27). Ceruloplasmin had a correlation coefficient of $r = 0.70$ with TCA-soluble Cu levels.

## DISCUSSION

### Performance - Feed Intake, Live Weight Gain

Feed consumption among treatment groups was not depressed by high sulphur content of the respective dietary treatments. Over the 118 day experiment, cattle on the high sulphur treatments consumed more feed than cattle fed the diet with the lowest sulphur content. This acceptability to the steers of high sulphur diets on a long term basis may be attributable to the incorporation of 3.0% molasses into the diet. Molasses was added to the diet to mask the repugnant taste associated

Table 26. Experiment 3. Correlation coefficients among Cu criteria of beef steers[1-3]

| Y/X | Liver Cu | Serum Cu | Ceruloplasmin | TCA | PR |
|---|---|---|---|---|---|
| Liver Cu | 1.00 | -0.18 | -0.24 | -0.31 | -0.30 |
| Serum Cu | - | 1.00 | 0.75 | 0.85 | -0.13 |
| Ceruloplasmin | - | - | 1.00 | 0.70 | 0.02 |
| TCA | - | - | - | 1.00 | 0.40 |
| PR | - | - | - | 0.40 | 1.00 |

[1]N = 80.

[2]X = Independent variable and Y = dependent variable.

[3]Liver Cu (ppm), serum Cu (µg/ml), ceruloplasmin (abs. at 540 mµ), TCA = TCA-soluble Cu (µg/ml), and PR = soluble Cu as a % of serum Cu.

Table 27. Experiment 3. A test of significance of difference between two correlation coefficients

| Method | Sample size (N) | r[1] | Z[2] | 1/(N-3) | D/S | Conclusion[4] |
|---|---|---|---|---|---|---|
| A | 80 | .84 | 1.221 | .013 | | |
| | | | | | 1.54 | N.S. |
| B | 80 | .75 | .973 | .013 | | |
| | | | D=.248 | S=.026 | | |

[1] r of method A = correlation coefficient between TCA-soluble Cu and total serum Cu.
r of method B = correlation coefficient between ceruloplasmin activity and total serum Cu.

[2] D = Absolute difference.
S = Sum.

[3] N.S. = Not significant (P>.05).
* = Significant (P<.05).

with high sulphur diets. In a similar 140-day trial, Pendlum et al. (1976) fed Holstein steers diets containing from 0.11% to 0.45% S and reported no reduced feed intakes. They also included 2.0% molasses in their diets.

Regardless of sulphur treatment, there were periodic depressions in feed intake among the cattle groups probably related to the hot-humid summer weather. A large proportion of the daily feed in the feed bunks was frequently left behind when temperatures above 32°C were recorded.

Weight gain among the cattle suffered during the summer, especially period IV. The lowest weight gains for most of the steers were reported in this period and two steers lost weight (Table 28A). Although it was hot and humid, the animals had access to shelter, and constant access to water and remained in a healthy condition.

Copper Status - Liver Cu

At the onset of the experiment, six steers had liver copper concentrations which were lower than 10 ppm wet weight. Eight additional animals could be classified as marginally deficient (11-18 ppm Cu in liver) and only two animals were copper adequate (above 25 ppm) (Table 30A). The copper deficient animals did not display any clinical symptoms associated with a copper deficiency such as unthriftiness, lameness, dry hair coat or discoloration of coat color (impossible to detect in mottled steers found in groups B, C and D). Rather, all copper deficient cattle and their marginally Cu deficient pen mates were lively, healthy animals. In an earlier study, Mills et al. (1976) also recognized a poor cor-

relation between copper concentration in the liver or blood and the clinical condition of copper deficiency.

By the end of the first period all but two of the steers had accumulated enough copper in the liver to be classified as copper adequate (Fig. 10). Throughout the experiment, all treatment groups continued to store copper in the liver. Steers on the lowest and highest sulphur treatments (groups A and D) tended to have larger increases in hepatic levels of copper than those consuming diets of moderate sulphur content (groups B and C).

At a low level of dietary sulphur (e.g. group A) molybdate may combine with copper in the gut to form the cupric molybdate complex. This compound is absorbed through the gut and is excreted unchanged in the urine (Huisingh et al. 1973). All excess (unbound) dietary copper is also absorbed but ultimately is stored in the liver. Under more moderate dietary sulphur conditions (e.g. treatments B and C), thiomolybdates are more likely to be formed in the rumen and react with copper in the gastrointestinal tract thus preventing Cu absorption. Alternatively, thiomolybdates can be absorbed into the blood stream and react systemically with blood Cu. By either route thiomolybdates can potentially render much copper unavailable for storage and for biological functions (Mills et al. 1979). Under such moderate dietary sulphur levels, less excess copper will be available for hepatic storage compared to low sulphur conditions. Finally, at high levels of dietary sulphur (Treatment D), sulphate and molybdate possibly compete (more vigorously) against each other for a common carrier essential for Mo and S transportation across membranes

(Huisingh et al. 1973). In the presence of high dietary sulphur, sulphate (which escapes reduction in the rumen) saturates intestinal and renal membrane carrier systems thereby reducing molybdenum absorption in the gut and reabsorption in the kidney tubules. Because of this reduction in Mo absorption and increase in Mo excretion respectively, more dietary copper is available for the ruminant. However, dietary copper may still be bound by sulphide formed in the rumen to produce insoluble CuS.

Copper Status - Serum Cu Parameters

Throughout the experiment, the mean levels of serum Cu in the various treatment groups could be classified as marginally deficient. Serum Cu levels rose during the first period and fell slightly for the rest of the experiment (Fig. 11). The initial rise in serum Cu among all treatment groups may be attributed to the consumption of diets containing a moderately high copper content (20 ppm) by cattle with low hepatic levels of Cu. The subsequent decreases in serum Cu levels among pens were possibly due to the formation and rapid excretion of cupric molybdate from the blood stream (Treatment A), formation of thiomolybdates that bind copper in the gut (Treatments B and C) or the formation of unabsorbable cupric sulphide also in the gut (Treatment D).

Changes in ceruloplasmin activity and TCA-soluble levels (absolute and percent of total serum Cu) were found among the four treatment groups throughout the experiment. Ceruloplasmin activity levels of the first three groups followed no particular pattern of periodic increases and

decreases (Fig. 12), and with the exception of period I, the differences of such levels among groups A, B and C were small. In contrast, the ceruloplasmin level of group D tended to drop (Fig. 12) continuously (until the end of period III) and to a greater magnitude. Likewise, TCA-soluble Cu levels among pens followed a similar pattern to total serum Cu with a tendency for steers on treatment D to have lower TCA-soluble Cu levels (Fig. 13).

At the beginning of the experiment it was also found that initial levels of serum Cu, ceruloplasmin activity and TCA-soluble Cu levels (including liver Cu) were consistently lower in group B (0.3% S) than in the other three groups. However, at the end of the four month experiment, the overall serum copper, ceruloplasmin activity and TCA-soluble Cu level (absolute) of group B increased whereas losses in the same parameters were expressed by the other three groups. In addition, an overall loss in the percentage of TCA-soluble Cu in total serum Cu was smaller in group B compared to losses of TCA-soluble Cu (% of total Cu) in the other three groups.

The initial lower levels of the serum Cu parameters in group B may be attributed to low mean hepatic Cu level found at the beginning of the experiment. Although not significantly different ($P>.05$) group B started the experiment with the lowest mean liver Cu levels of the four groups. One may speculate that since very low blood copper levels are maintained at the expense of liver copper reserves, serum copper levels and related parameters of ceruloplasmin activity and TCA-soluble Cu in group B would also be lower compared to the other three groups (Claypool et al. 1975).

Simpson et al. (1982) hypothesized that low hepatic reserves of copper are conserved by homeostatic regulation of endogenous losses and therefore, the retention of dietary Cu in marginally Cu-deficient animals will be greater than animals with adequate copper reserves. Similarly, in the present experiment, the overall increase in serum Cu parameters in group B compared to the overall losses in the other three groups are possibly due to a larger retention of dietary copper in the serum of group B. Those animals with a lower serum Cu may be able to reduce serum Cu losses (e.g. ceruloplasmin degradation) and thus conserve copper which contributes to an increase in the serum copper parameters.

Most of the differences in serum ceruloplasmin activity and TCA-soluble Cu levels over time and among treatment groups (regardless of significance), occurred with concurrent changes in total serum Cu. About 56% and 70% ($r^2$ x 100, r = .75, r = .84, not significantly different at P>.05) of the differences in ceruloplasmin activity and TCA-soluble Cu levels respectively could be explainable by differences in total copper concentrations in the serum (Snedecor and Cochran 1980). Therefore, changes in levels of serum Cu components may be attributable partially to changes in total serum Cu by external factors (i.e. formation of CuS in the gut) and partially to the pattern of distribution in serum Cu by internal factors (i.e. absorption of thiomolybdates into the blood stream and alterations in excretion.

The results found for the serum Cu and related parameters are similar to those reported by Suttle (1974b), who demonstrated that the

addition of sulphur to the diets of ewes decreased the response of plasma Cu to dietary copper supplementation. Suttle (1974b) hypothesized that such reactions were due to the formation of insoluble CuS in the gut. This proposed mechanism could explain some of the behavior of serum Cu parameters by animals consuming the highest level of dietary sulphur (treatment D).

Indicators of Copper Status

Serum copper parameters seemed to best reflect the influence of the various dietary sulphur treatments of beef yearlings. This influence was illustrated partly through changes in total serum Cu concentration and also through the distribution of Cu in the serum (i.e. ceruloplasmin and TCA-soluble Cu). Since these three criteria were highly correlated, one can verify a large portion of the changes in one serum Cu criterion by observing similar alterations in another serum Cu criterion. However, a change in ceruloplasmin activity or TCA-soluble Cu level does not necessarily mean a change in total serum Cu, although these components account for a very large portion of Cu in the serum.

At the beginning of the experiment, many animals were classified as severely copper deficient on the basis of liver Cu, but these animals failed to display any clinical characteristics associated with a copper deficiency. This phenomenon is attributed to the poor association between liver Cu and the clinical condition of a copper deficiency (Suttle 1976, Mills et al. 1976).

Although deficient liver Cu levels were found in the absence of

clinical signs of a copper deficiency, liver Cu was still an indicator of copper accumulation in the body during the experiment. However serum Cu and liver Cu did not reflect the effects of sulphur upon the copper state of the cattle. Hepatic Cu levels were found to be poorly correlated with total serum Cu ($r = -.18$). Since most serum samples for the duration of the experiment recorded a copper level of above 0.50 µg/ml, a poor correlation coefficient between liver Cu and serum Cu are in agreement with the suggestions of Claypool et al. (1975). In contrast, there was a tendency for initial levels of serum Cu in treatment B to be associated with corresponding low initial hepatic Cu levels also found in treatment B.

## CONCLUSIONS

1. Live weight gain, ADG and dressed carcass weight among steers were not significantly ($P>.05$) affected by dietary treatment.
2. Feed consumption was not significantly ($P>.05$) affected by dietary treatment. On a long term basis, each pen of animals accepted their assigned dietary S rations containing molasses.
3. Liver Cu, total serum Cu and TCA-soluble Cu levels among treatment groups were not significantly ($P>.05$) altered by dietary treatment. On the other hand, S treatments did significantly alter ($P<.05$) ceruloplasmin activity among treatment groups.
4. Liver Cu levels were elevated ($P<.01$) throughout all four periods of the experiment. Likewise, all serum Cu parameters changed with time on treatment (total serum Cu, $P<.01$; ceruloplasmin

activity, $P<.05$; TCA-soluble Cu, $P<.01$); however, most serum Cu levels increased initially and then declined throughout the remainder of the experiment.

5. Serum Cu parameters, best reflected the influence of various dietary sulphur treatments upon copper status of beef yearlings. Liver Cu was still employed as a measure of copper accumulation in the body.

# SUMMARY

Three experiments were designed to examine the interrelationship between Cu, Mo and S.

The theme of the first experiment is based upon the concept that the concentration of copper and molybdenum in a 2:1 ratio in the diet is an estimate of the dietary copper requirement for cattle (Miltimore and Mason 1971). Contrary to the suggestions of Miltimore and Mason (1971), the absolute concentrations of dietary copper and molybdenum within this ratio are also viewed as important, when assessing such copper needs. Therefore, copper and molybdenum were supplemented in the diet in order to study the effects of four various concentrations of copper and molybdenum in a 2:1 ratio upon the performance and copper parameters in beef cattle.

The level of hepatic copper among the treatment groups was significantly ($P<.05$) affected by treatment, whereas overall changes in serum copper parameters were not substantially ($P>.05$) different among the treatment groups. Throughout the experiment, cattle among the four groups showed elevated liver Cu levels as well as a tendency to post small gains in serum Cu (with the exception of one group) and ceruloplasmin activity criteria. The magnitude of such hepatic copper accumulation tended to be positively related to the copper concentration of the treatment diets. From these results, one may conclude that not all the dietary copper in a Cu/Mo ratio of 2:1 binds molybdenum to form the biologically unavailable Cu:Mo complex in the animal's digestive tract. Furthermore, this unbound copper is possibly greater in cattle

consuming diets with higher copper concentrations compared to bovine groups consuming a marginal level of copper in the diet, regardless of the 2:1 Cu/Mo ratio of both diets. Such extra copper is first used to meet the animal's copper requirement and any excess is stored in the liver.

In the first experiment, the effects of dietary sulphur upon copper status in beef cattle were largely ignored. Therefore, second and third experiments were independently conducted to test the effects of four different levels of sulphur in the diet upon liver Cu and serum Cu parameters.

The second experiment which followed a latin square design was performed as a preliminary study for the third (second sulphur) experiment. The main objective of this study was to provide some insight as to feed palatability and intake by cattle of diets supplemented with sodium sulphate and also to preview the effects of sulphur on measured copper criteria.

The results indicate that feed consumption was not significantly ($P>.05$) affected by sulphur additions to the diet. Because of possible sampling errors, the validity concerning the effect of sulphur upon liver Cu and serum Cu parameters was questioned. Regardless, liver Cu, ceruloplasmin activity and TCA-soluble levels rose by the end of the experiment, whereas total serum Cu levels remained relatively stable.

Feed intake by the steers was not depressed by the sodium sulphate content of the diets in Experiment 2, and as a result the same level of sulphur supplementation was maintained in the four treatment diets of the third experiment. Since these treatment diets were to be fed on a

continuous basis (compared to a series of diet rotations in Experiment 2), molasses was added to the diets to insure satisfactory palatability of the feed.

Total feed consumption among the treatment groups in the third experiment did not differ significantly ($P>.05$). Any depression in feed intake in the intervening periods was not attributed to dietary sulphur additions but rather to environmental factors such as a long hot and humid summer.

Dietary S treatments were also found to have no significant ($P>.05$) effect on liver Cu, serum Cu, TCA-soluble Cu levels but significantly ($P<.05$) affected ceruloplasmin levels among the four cattle groups. Any differences recognized with respect to liver Cu levels among the S-groups were in agreement with many aspects of the Cu-Mo-S interactions, namely: Cu-Mo complexing, the thiomolybdate theory, Mo-S competition, and CuS formation. Furthermore, all four treatment groups had substantial elevation of hepatic Cu levels throughout the experiment. Similarly, the effect of sulphur treatments upon serum Cu parameters were also explained by the same aspects of the Cu-Mo-S interactions. Since ceruloplasmin activity and TCA-soluble Cu levels are highly correlated with total serum Cu, many of the differences (regardless of significance) in these former two copper parameters among treatment groups and experimental periods were attributed to changes in the level of total serum Cu. One might hypothesize that if serum Cu levels were decreased due to complexing of CuS in the gut, ceruloplasmin activity and TCA-soluble Cu levels would subsequently decline. However, some of the differences in

these two serum criteria among treatment groups were possibly due to the systemic actions of serum Cu-binding thiomolybdates.

In all three experiments, the choice of an adequate indicator of copper status in beef yearlings was constantly being scrutinized. Although, the copper status of all steers was initially classified on the basis of liver and total serum copper levels, the primary objective of a good copper indicator was to reflect the effects of dietary treatment upon copper concentrations in the body. In the first experiment, the liver Cu parameter best examined the effects of different concentrations of Cu and Mo in a 2:1 ratio in the diet upon copper status in beef steers. In the third experiment, serum copper parameters tended to reflect the effects of sulphur upon beef cattle copper status. The choice of a good indicator of copper status in the second experiment was deemed inconclusive because of sampling difficulties.

# LITERATURE CITED

Amer, M.A., G.J. St. Laurent and G.J. Brisson. 1973. Supplemental copper and selenium for calves: effects upon ceruloplasmin activity and liver copper concentration. Can. J. Physiol. Pharmacol. 51:649-653.

A.O.A.C. 1975. Official methods analysis. (8th ed.). Association of Official Analytical Chemists. Washington, D.C.

A.O.A.C. 1980. Official methods analysis. (13th ed.). Association of Official Analytical Chemists. Washington, D.C.

Bertelsen, A. 1982. Beef: Trace element deficiencies lower cattle reproduction. Feedstuffs 55:21.

Bingley, J.B. 1974. Effects of high doses of molybdenum and sulphate on the distribution of copper in plasma and in blood of sheep. Aust. J. Agric. Res. 25:467-747.

Bingley, J.B. and N. Anderson. 1972. Clinically silent hypocuprosis and the effect of molybdenum loading on beef calves in Gippsland, Victoria. Aust. J. Agric. Res. 23:885-904.

Bingley, J.B. and A.T. Dick. 1969. The pH optimum for ceruloplasmin oxidase activity in the plasma of several species of animal. Clini. Chim. Acta. 25:480-482.

Bingley, J.B. and J.H. Dufty. 1972. Distribution of copper in the tissues of the bovine neonate and dam. Res. Vet. Sci. 13:8-14.

Boila, R.J., T.J. Devlin, R.A. Drysdale and L.E. Lillie. 1984a. Geographical variation in the copper and molybdenum content of forages grown in northwestern Manitoba. Can. J. Anim. Sci. 64:899-918.

Boila, R.J., T.J. Devlin, R.A. Drysdale and L.E. Lillie. 1984b. The severity of hypocupremia in selected herds of beef cattle in northwestern Manitoba. Can. J. Anim. Sci. 64:919-936.

Bremner, I. 1976. The relationship between the zinc status of pigs and the occurrence of copper- and Zn-binding proteins in liver. Br. J. Nutr. 35:245-252.

Bremner, I. and R.B. Marshall. 1974a. Hepatic copper- and zinc-binding proteins in ruminants. 1. Distribution of Cu and Zn among soluble proteins of livers of varying Cu and Zn content. Br. J. Nutr. 32:283-291.

Bremner, I. and R.B. Marshall. 1974b. Hepatic copper- and zinc-binding proteins in ruminants. 2. Relationship between Cu and Zn concentrations and the occurrence of a metallothionein-like fraction. Br. J. Nutr. 32:293-300.

Brenmer, I. and C.F. Mills. 1981. Absorption, transport and tissue storage of essential trace elements. Phil. Trans. R. Soc. Lond. 294:75-89.

Bremner, I. and B.W. Young. 1978. Effects of dietary molybdenum and sulphur on the distribution of copper in plasma and kidneys of sheep. Br. J. Nutr. 39:325-336.

Bull, R.C. 1980. Copper. Animal Nutrition and Health. p. 32-34.

Campbell, C.M., J.C. Nolan, R.W. Stanley and O. Wayman. 1976. Effects of molybdenum and copper relationship on early weaned calves. In: W.R. Chappell and K.K. Petersen (Ed.) Molybdenum in the Environment. Marcel and Dekker, Inc., New York.

Chapman, H.L., Jr., D.H. Cox, C.E. Haines and G.K. Davis. 1963. Evaluation of the liver biopsy technique for mineral nutrition studies with beef cattle. J. Anim. Sci. 22:733-737.

Clawson, W.J., A.L. Lesperance, V.R. Bohman and D.C. Layhee. 1972. Interrelationship of dietary molybdenum and copper on growth and tissue composition of cattle. J. Anim. Sci. 34:516-520.

Claypool, D.W., F.W. Adams, H.W. Pendell, N.A. Hartman, Jr. and J.F. Bone. 1975. Relationship between the level of copper in the blood plasma and liver of cattle. J. Anim. Sci. 41:911-914.

Cook, G.A., A.L. Lesperance, V.R. Bohman and F.H. Jensen. 1966. Interrelationship of molybdenum and certain factors to the development of the molybdenum toxicity syndrome. J. Anim. Sci. 25:96-101.

Cunningham, H.M., J.M. Brown and A.E. Edie. 1953. Molybdenum poisoning of cattle in the Swan River Valley of Manitoba. Can. J. Agr. Sci. 33:254-260.

Dick, A.T., D.W. Dewey and J.M. Gawthorne. 1975. Thiomolybdates and the copper-molybdenum-sulphur interaction in ruminant nutrition. J. Agric. Sci. 85:567-568.

DiSilvestro, R.A. and R.J. Cousins. 1983. Physiological ligands for copper and zinc. Ann. Rev. Nutr. 3:261-288.

Dowdy, R.P. and G. Matrone. 1968a. Copper-molybdenum interaction in sheep and chicks. J. Nutr. 95:191-196.

Dowdy, R.P. and G. Matrone. 1968b. A copper-molybdenum complex: Its effects and movement in the piglet and sheep. J. Nutr. 95:197-201.

Dreosti, I.E. and G.V. Quicke. 1968. Blood copper as an indicator of copper status with a note on serum proteins and leucocyte counts in copper-deficient rats. Br. J. Nutr. 22:1-7.

Engel, R.W., W.A. Hardison, R.F. Miller, N.O. Price and J.T. Huber. 1964. Effect of copper intake on concentration in body tissue and on growth, reproduction and production in dairy cattle. J. Anim. Sci. 23:1160-1163.

Erdman, J.A., R.J. Ebens and A.A. Case. 1978. Molybdenosis: A potential problem in ruminants grazing on coal mine spoils. J. Range Management 31:34-36.

Erwin, E.S., I.A. Dyer, T.O. Meyer and K.W. Scott. 1956. Uses of aspiration of biopsy technique. J. Anim. Sci. 15:428-434.

Evans, G.W. 1973. Copper homeostasis in the mammalian system. Physiological Reviews 53:535.

Fischer, P.W.F., A. Giroux and M.R. L'Abbé. 1981. The effect of dietary zinc on intestinal copper absorption. Amer. J. Clin. Nutr. 34:1670-1675.

Friedan, E. 1968. The biochemistry of copper. Sci. Am. 218:103-113.

Gawthorne, J.M. and C.J. Nader. 1976. The effect of molybdenum on the conversion of sulphate to sulphide and microbial-protein-sulphur in the rumen of sheep. Br. J. Nutr. 35:11-23.

Givens, D.I. and J.R. Hopkins. 1981. The effect of copper therapy on the growth rate and blood composition of young cattle. J. agric. Sci. Camb. 97:497-505.

Goodrich, R.D., T.S. Kahlon, D.E. Pomp and D.P. Cooper. 1978. Sulphur in ruminant nutrition. National Feed Ingredients Association. West Des Moines, Iowa.

Gorz, H.J. and W.K. Smith. 1973. Sweet clover. In: M.E. Heath, D.S. Metcalfe and R.F. Barnes (Ed.) Forages. Iowa State University Press, Ames, Iowa.

Hamm, J.W., J.R. Bettany and E.H. Halstead. 1973. A soil test for sulphur and interpretative criteria for Saskatchewan. Comm. in Soil Science and Plant Anal. 4:219-231

Hansard, S.L. 1983. Micronutrients for ruminant animals. Nutrition Abstracts and Reviews. Vol. 53, No. 1.

Holtzman, N.A. and B.M. Gaumnitz. 1970. Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma. J. Biol. Chem. 245:2354-2358.

Hubbs, R. and F.W. Oehme. 1982. Understanding chronic copper poisoning in sheep. Bovine Practice, Vol. 3, No. 5:15-22.

Huber, J.T., N.O. Price and R.W. Engel. 1971. Response of lactating dairy cows to high levels of dietary molybdenum. J. Anim. Sci. 32:364-367.

Huisingh, J., G.G. Gomez and G. Matrone. 1973. Interactions of copper, molybdenum and sulfate in ruminant nutrition. Federation Proceedings 32:1921-1924.

Huisingh, J. and G. Matrone. 1976. Interactions of molybdenum in animal nutrition. In: W.R. Chappell and K.K. Petersen (Ed.) Molybdenum in the Environment. Marcel and Dekker, Inc., New York.

Huisingh, J., D.C. Milholland and G. Matrone. 1975. Effect of molybdate on sulfide production from methionine and sulfate by ruminal microorganisms of sheep. J. Nutrition 105:1199-1205.

Humphries, W.R., B.W. Young, M. Phillippo and I. Bremner. 1981. The effect of iron and molybdenum on copper metabolism in cattle. Proc. Nutr. Soc. 40:68A.

Irwin, M.R., W.C. Bergin, T.R. Sawa, L.B. McKinney and H. Kimura. 1979. Poor growth performance associated with hypocupremia in Hawaiian feedlot cattle. J. Am. Vet. Med. Assoc. 174:590-593.

Ishida, N., T. Yoshikawa and K. Kawashima. 1982. Effects of oral and intravenous administrations of molybdenum and sulphate on distribution of copper in blood of sheep. Memoirs of the College of Agriculture, Kyoto Univ. 119:23-34.

Jamieson, S. and R. Allcroft. 1950. Copper pine of calves. Brit. J. Nutr. 4:16-31.

Keeton, W.T. 1972. Biological Science. W.W. Norton and Company, Inc. New York.

Kelleher, C.A., M. Ivan, M. Lamond and J. Mason. 1983. The absorption of labelled molybdenum compounds in sheep fitted with re-entrant cannulae in the ascending duodenum. J. Comp. Path. 93:83-92.

Lamand, M., C. Lab, J.C. Tressel and J. Mason. 1980. Biochemical parameters useful for the diagnosis of mild molybdenum in sheep. Ann. Rech. Vet. 11:141-145.

Linder, M.C., P.A. Houle, E. Isaacs, J.R. Moor and L.E. Scott. 1979. Copper regulation of ceruloplasmin in copper-deficient rats. Enzyme 24:23-35.

Lorentz, P.P. and F.M. Gibb. 1975. Caeruloplasmin activity as an indication of plasma copper levels in sheep. New Zealand Veterinary Journal 23:1-3.

Maclean, N. 1978. Haemoglobin. Camelot Press Ltd., Southampton.

Mason, J., M. Lamand and C.A. Kelleher. 1980. The fate of $^{99}$Mo-labelled sodium tetrathiomolybdate after duodenal administration in sheep: the effect on caeruloplasmin (EC 1.16.3.1) diamine oxidase activity and plasma copper. Br. J. Nutr. 43:515-523.

Mason, J., M. Lamand and C.A. Kelleher. 1982. The effects of duodenal infusion of tri- and dithiomolybdate on plasma copper and on the diamine oxidase activity of caeruloplasmin (EC 1.16.3.1) in sheep. J. Comp. Path. 92:509-518.

Mason, J., M. Lamand, J.C. Tressol and C. Lab. 1978. The influence of dietary sulphur, molybdate and copper on the absorption, excretion and plasma fraction levels of $^{99}$Mo in sheep. Ann. Rech. Vet. 9:577-586.

McDowell, L.R., J.H. Conrad and G.L. Ellis. 1983. Mineral deficiencies, imbalances and diagnosis: Part I. Feedstuffs 56:31-40.

Miller, W.J. 1974. New concepts and developments in metabolism and homeostasis of inorganic elements in dairy cattle. J. Dairy Sci. 58:1549-1560.

Mills, C.F. 1979. Trace elements in animals. Phil. Trans. R. Soc. Lond. 288:51-63.

Mills, C.F., A.C. Dalgarno and G. Wenham. 1976. Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency. Br. J. Nutr. 35:309-330.

Miltimore, J.E. and J.L. Mason. 1971. Copper to molybdenum ratio and molybdenum and copper concentrations in ruminant feeds. Can. J. Anim. Sci. 51:193-200.

Miltimore, J.E., J.L. Mason, J.L. McArthur and R.B. Carson. 1964. Ruminant mineral nutrition. The effect of copper injections on weight gains and haemoglobin levels of cattle pastured on groundwater soils in the British Columbia interior. Can. J. Comp. Med. Vet. Sci. 28:108-112.

National Research Council. 1976. Nutrient requirements of beef cattle. (5th edition). National Academy of Sciences, Washington, D.C.

Nikolic, J.A., M. Jovanovic, R. Andric, D. Djordjevic and J. Krsmanovic. 1983. Examination of the effect of copper and molybdenum on microbial protein synthesis in rumen contents using $^{35}S$. Int. J. Appl. Radiat. Isot. 34:809-812.

O'Cuill, T., A.F. Hamilton and D.A. Egan. 1970. Copper distribution in the liver. Irish Vet. Journal 24:21-25.

Osaki, S., D.A. Johnson and E. Frieden. 1966. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J. Biol. Chem. 241:2746-2751.

Peck, H.D. 1959. The ATP-dependent reduction of sulfate with hydrogen in extracts of desulfovibrio desulfuricans. Biochemistry 45:701-708.

Pendlum, L.C., J.A. Boling and N.W. Bradley. 1976. Plasma and ruminal constituents and performance of steers fed different nitrogen sources and levels of sulphur. J. Anim. Sci. 43:1307-1314.

Penumarthy, L. 1978. Molybdenum toxicosis in cattle. Veterinary and Human Toxicology 20:11-20.

Phillippo, M. and D.S. Graca. 1983. Biliary copper secretions in cattle. Proc. Nutr. Soc. Abstract 42:46A.

Puls, R. 1981. Veterinary trace mineral deficiency and toxicity information. Information Services, Agriculture Canada, Ottawa.

Rusoff, L.L. 1981. Mineral deficiencies and toxicities in dairy cattle. Feedstuffs 53:29-30.

Rice, E.W. 1962. Standardization of ceruloplasmin activity in terms of international enzyme units. Analytical Biochemistry 3:452-456.

Rice, E.W. 1963. Ceruloplasmin assay in serum: standardization of ceruloplasmin activity in terms of international enzyme units. In: D. Seligsen (Ed.) Standard Methods of Clinical Chemistry. Vol. 4. Acad. Press, New York.

Saylor, W.W., F.D. Morrow and R.M. Leach, Jr. 1980. Copper- and zinc-binding proteins in sheep liver and intestine: effects of dietary levels of the metals. J. Nutr. 110:460-468.

Siegel, L.M. and K.J. Monty. 1961. A mechanism for the copper-molybdenum interrelationship. II. Response of liver sulfide oxidase activity to nutritional factors. J. Nutrition 74:167-170.

Simpson, A., C.F. Mills and I. McDonald. 1982. Tissue copper retention or loss in young growing cattle. In: J.M. Gawthorne, J. McC. Howell and C.L. White (Ed.) Trace Element Metabolism in Man and Animals. Springer-Verlag, Berlin.

Smart, M.E., J. Gudmundson, R.P. Brockman, N. Cymbaluk and C. Doige. 1980. Copper deficiency in calves in north central Manitoba. Can. Vet. J. 21:349-352.

Smith, B.P., G.L. Fisher, P.W. Poulos and M.R. Irwin. 1975. Abnormal bone development and lameness associated with secondary copper deficiency in young cattle. JAVMA 166:682-688.

Snedecor, G.W. and W.G. Cochran. 1980. Statistical Methods. 7th Ed. Iowa State University Press, Ames, Iowa.

Sourkes, T.L., K. Lloyd and H. Birnbaum. 1968. Inverse relationship of hepatic copper and iron concentrations in rats fed deficient diets. Can. J. Biochem. 46:267-271.

Suttle, N.F. 1974a. Recent studies of copper-molybdenum antagonism. Proc. Nutr. Soc. 33:299-305.

Suttle, N.F. 1974b. Effects of organic and inorganic sulphur on the availability of dietary copper to sheep. Br. J. Nutr. 32:559-568.

Suttle, N.F. 1975. The role of organic sulphur in the copper-molybdenum-S interrelationship in ruminant (sheep) nutrition. Br. J. Nutr. 34:411-420.

Suttle, N.F. 1976. The detection and prevention of trace deficiencies in animal husbandry systems. Chemistry and Industry 44:559-562.

Suttle, N.F. 1979. Changes in hepatic copper stores in pre-ruminant and ruminant lambs given ammonium tetra-thiomolybdate in their diets. Abstract, Proc. Nutr. Soc. 58A.

Suttle, N.F. 1980. The role of thiomolybdates in the nutritional interactions of copper, molybdenum and sulfur: fact or fantasy? Annuals of the New York Academy of Sciences 355:195-207.

Suttle, N.F. 1983. Use of erthrocyte copper: zinc superoxide dimutase activity and hair or fleece copper concentrations in the diagnosis of hypocuprosis in ruminants. Res. Vet. Sci. 35:47-52.

Suttle, N.F. and K.W. Angus. 1976. Experimental copper deficiency in the calf. J. Comp. Path. 86:595-608.

Suttle, N.F., K.W. Angus, D.I. Nisbet and A.C. Field. 1972. Osteoporosis in copper-depleted lambs. J. Comp. Path. 82:93-97.

Suttle, N.F. and A.C. Field. 1983. Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. J. Comp. Path. 93:379-389.

Stanier, R.Y., E.A. Adelberg and J. Ingraham (ed.). 1976. The Microbial World. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Tephly, T.R., G. Wagner, R. Sedman and W. Piper. 1978. Effects of metals on heme biosynthesis and metabolism. Fed. Proceedings 37:35-39.

Thompson, R.H. and W.J. Blanchflower. 1971. Wet-ashing apparatus to prepare biological materials for atomic absorption spectrophotometry. Lab. Pract. 20:859-861.

Thompson, R.H. and J.R. Todd. 1976. The copper status of beef cattle in northern Ireland. Br. J. Nutr. 36:299-303.

Thornton, I., G.F. Kershaw and M.K. Davis. 1972a. An investigation in copper deficiency in cattle in the Southern Pennines. I. Identitification of suspect areas using geochemical reconnaissance followed by blood copper surveys. J. Agr. Sci. Camb. 78:157-163.

Thornton, I., G.K. Kershaw and M.K. Davis. 1972b. An investigation of copper deficiency in cattle in the Southern Pennines. II. Response to copper supplementation. J. Agr. Sci. Camb. 78:165-171.

Todd, J.R. 1970. A survey of the copper status of cattle using copper oxidase (ceruloplasmin) activity of blood serum. In: Trace Element Metabolism in Animals, pp. 448-449 (C.F. Mills, editor). Edinburgh and London: E. and W. Livingstone.

Underwood, E.J. 1976. Molybdenum in animal nutrition. In: W.R. Chappell and K.K. Peterson (Ed.). Molybdenum in the Environment. Marcel and Dekker, Inc., New York.

Underwood, E.J. 1977. Copper. In: Trace Elements in Human and Animal Nutrition. 4th edit. :123-127. Academic Press, Inc. New York, NY.

Vanderveen, J.E. and H.A. Keever. 1964. Effects of molybdenum and sulfate sulfur on metabolism of copper in dairy cattle. J. Anim. Sci. 47:1224-1230.

Ward, G.M. 1978. Molybdenum toxicity and hypocuprosis in ruminants: A review. J. Anim. Sci. 46:1078-1085.

Wilson, L.G. and R.S. Bandarski. 1958. Enzymatic reactions involving sulfate, sulfite, selenate and molybdate. J. Biol. Chem. 233:975-981.

# APPENDIX I

Table 1A. Experiment 1. Raw data - Initial body wt. (kg)

| Treatment | Animal | Initial weight | Mean | Std. Dev.[1] |
|---|---|---|---|---|
| A | 103 | 364 | 362.0 | 16.81 |
| | 100 | 356 | | |
| | 095 | 384 | | |
| | 073 | 344 | | |
| B | 105 | 354 | 358.5 | 24.97 |
| | 096 | 332 | | |
| | 004 | 392 | | |
| | 069 | 356 | | |
| C | 101 | 352 | 354.5 | 16.76 |
| | 013 | 332 | | |
| | 081 | 370 | | |
| | 001 | 364 | | |
| D | 106 | 364 | 363.5 | 28.58 |
| | 016 | 328 | | |
| | 074 | 398 | | |
| | 083 | 364 | | |

[1]Std. Dev. = standard deviation.

Table 2A. Experiment 1. Raw data - body wt. (kg)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 364 | 370 | 394 | 416 | 462 |
| | 100 | 356 | 394 | 446 | X | X |
| | 095 | 384 | 410 | 442 | 456 | 522 |
| | 073 | 344 | 372 | 403 | 432 | 498 |
| B | 105 | 354 | 364 | 389 | 406 | 450 |
| | 096 | 332 | 352 | 386 | 406 | 486 |
| | 004 | 392 | 414 | 452 | X | X |
| | 069 | 356 | 376 | 402 | 436 | 502 |
| C | 101 | 352 | 379 | 404 | 458 | 484 |
| | 013 | 332 | 352 | 388 | 436 | 478 |
| | 081 | 370 | 400 | 439 | 484 | 526 |
| | 001 | 364 | 392 | 424 | 464 | 490 |
| D | 106 | 364 | 384 | 408 | 458 | 474 |
| | 016 | 328 | 353 | 377 | 418 | 468 |
| | 074 | 398 | 415 | 450 | 498 | 546 |
| | 083 | 364 | 377 | 416 | X | X |

[1] X = missing value.

[2] T = treatment.

Table 3A. Experiment 1. Raw data - performance[1]

| T[2] | Animal | Final wt. | Gain (kg) | ADG (kg/d) | Dressed (kg) | % of live wt. | Grade |
|---|---|---|---|---|---|---|---|
| A | 103 | 562 | 98 | 0.81 | 273.3 | 51.34 | A1 |
| | 100 | X | X | X | X | X | B1 |
| | 095 | 522 | 138 | 1.14 | 260.0 | 49.81 | B1 |
| | 073 | 498 | 154 | 1.27 | 254.5 | 51.11 | A1 |
| B | 105 | 450 | 96 | 0.79 | 244.5 | 54.34 | A1 |
| | 096 | 486 | 154 | 1.27 | 240.9 | 49.57 | A1 |
| | 004 | X | X | X | X | X | B1 |
| | 069 | 502 | 146 | 1.21 | 257.3 | 51.25 | A1 |
| C | 101 | 484 | 132 | 1.09 | 256.4 | 52.97 | A2 |
| | 013 | 478 | 146 | 1.21 | 233.6 | 48.87 | A1 |
| | 081 | 526 | 156 | 1.29 | 267.3 | 50.81 | A1 |
| | 001 | 490 | 126 | 1.04 | 261.8 | 53.43 | A1 |
| D | 106 | 474 | 110 | 0.91 | 250.9 | 52.93 | A1 |
| | 016 | 468 | 140 | 1.16 | 247.3 | 52.84 | A1 |
| | 074 | 546 | 148 | 1.22 | 286.4 | 52.54 | A1 |
| | 083 | X | X | X | X | X | X |

[1]X = missing value.

[2]T = treatment.

[3]Based on 121 days.

Table 4A. Experiment 1. Raw data - liver Cu (mg/kg, wet basis)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 34.66 | 27.93 | 40.35 | 43.07 | 53.49 |
| | 100 | 39.42 | 49.94 | 39.36 | X | X |
| | 095 | 23.15 | 23.76 | 56.34 | 63.29 | 79.39 |
| | 073 | 53.90 | 58.17 | 83.28 | 119.77 | 80.50 |
| B | 105 | 38.10 | 48.61 | 77.18 | 96.39 | 104.54 |
| | 096 | 33.08 | 39.93 | 53.11 | 80.80 | 106.28 |
| | 004 | 28.04 | 53.44 | 45.01 | X | X |
| | 069 | 33.89 | 59.39 | 70.87 | 101.79 | 97.93 |
| C | 101 | 18.81 | 65.21 | 81.17 | 85.57 | 83.66 |
| | 013 | 31.57 | 46.11 | 71.16 | 99.68 | 100.19 |
| | 081 | 38.64 | 49.59 | 76.37 | 106.31 | 144.24 |
| | 001 | 25.68 | 47.13 | 70.70 | 85.94 | 92.48 |
| D | 106 | 17.91 | 51.29 | 101.84 | 96.66 | 138.06 |
| | 016 | 42.82 | 79.90 | 179.31 | 206.20 | 214.63 |
| | 074 | 19.96 | 65.48 | 89.68 | 122.41 | 152.71 |
| | 083 | 43.92 | 113.58 | 74.98 | X | X |

[1] X = missing value.

[2] T = treatment.

Table 5A. Experiment 1. Raw data - serum Cu (µg/ml, at biopsy)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 0.973 | 0.758 | 0.925 | 0.943 | 0.833 |
| | 100 | 0.905 | 0.833 | 0.998 | X | X |
| | 095 | 0.780 | 0.820 | 0.680 | 0.813 | 0.733 |
| | 073 | 0.655 | 0.570 | 0.665 | 0.747 | 0.697 |
| B | 105 | 0.645 | 0.648 | 0.808 | 0.913 | 0.813 |
| | 096 | 0.760 | 0.795 | 0.643 | 0.840 | 1.000 |
| | 004 | 0.753 | 0.743 | 0.718 | X | X |
| | 069 | 0.855 | 0.788 | 0.755 | 0.843 | 0.743 |
| C | 101 | 0.895 | 0.805 | 0.740 | 0.737 | 0.803 |
| | 013 | 0.678 | 0.685 | 0.613 | 0.637 | 0.733 |
| | 081 | 0.805 | 0.813 | 0.695 | 0.937 | 0.927 |
| | 001 | 0.843 | 0.798 | 0.758 | 0.820 | 0.900 |
| D | 106 | 1.023 | 0.828 | 0.970 | 1.020 | 1.193 |
| | 016 | 0.835 | 0.780 | 1.138 | 1.520 | 0.717 |
| | 074 | 0.998 | 0.778 | 0.643 | 0.850 | 1.120 |
| | 083 | 0.953 | 0.803 | 1.040 | X | X |

[1] X = missing value.

[2] T = treatment.

Table 6A. Experiment 1. Raw data - ceruloplasmin (abs. at 540 mμ, at biopsy)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 0.126 | 0.153 | 0.069 | 0.182 | 0.156 |
| | 100 | 0.179 | 0.150 | 0.083 | X | X |
| | 095 | 0.145 | 0.117 | 0.119 | 0.118 | 0.129 |
| | 073 | 0.123 | 0.078 | 0.091 | 0.154 | 0.125 |
| B | 105 | 0.108 | 0.063 | 0.058 | 0.159 | 0.142 |
| | 096 | 0.155 | 0.093 | 0.039 | 0.131 | 0.174 |
| | 004 | 0.128 | 0.073 | 0.094 | X | X |
| | 069 | 0.137 | 0.094 | 0.099 | 0.101 | 0.098 |
| C | 101 | 0.133 | 0.170 | 0.054 | 0.120 | 0.158 |
| | 013 | 0.118 | 0.081 | 0.033 | 0.097 | 0.125 |
| | 081 | 0.129 | 0.021 | 0.132 | 0.106 | 0.194 |
| | 001 | 0.139 | 0.016 | 0.100 | 0.103 | 0.144 |
| D | 106 | 0.123 | 0.113 | 0.067 | 0.160 | 0.200 |
| | 016 | 0.136 | 0.162 | 0.079 | 0.200 | 0.167 |
| | 074 | 0.126 | 0.013 | 0.151 | 0.114 | 0.117 |
| | 083 | 0.123 | 0.118 | 0.126 | X | X |

[1] X = missing value.

[2] T = treatment.

Table 7A. Experiment 1. Raw data - serum Cu (µg/ml, 3 days after biopsy)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 0.915 | 1.135 | 0.890 | 0.958 | 0.890 |
| | 100 | 0.916 | 1.073 | 0.989 | X | X |
| | 095 | 0.849 | 0.745 | 0.698 | 0.740 | 0.673 |
| | 073 | 0.668 | 0.700 | 0.678 | 0.705 | 0.617 |
| B | 105 | 0.660 | 0.675 | 0.780 | 0.850 | 0.795 |
| | 096 | 0.880 | 0.918 | 0.730 | 0.755 | 0.660 |
| | 004 | 0.810 | 0.805 | 0.865 | X | X |
| | 069 | 0.881 | 0.823 | 0.848 | 0.808 | 0.688 |
| C | 101 | 0.798 | 0.908 | 0.885 | 0.808 | 0.746 |
| | 013 | 0.583 | 0.690 | 0.738 | 0.690 | 0.776 |
| | 081 | 0.800 | 0.942 | 0.900 | 0.950 | 0.896 |
| | 001 | 0.990 | 0.863 | 0.785 | 0.815 | 0.843 |
| D | 106 | 0.813 | 0.963 | 0.958 | 1.190 | 0.958 |
| | 016 | 0.838 | 1.300 | 1.575 | 1.555 | 1.320 |
| | 074 | 0.715 | 0.785 | 0.910 | 0.885 | 0.746 |
| | 083 | 0.733 | 0.980 | X | X | X |

[1] X = missing value.

[2] T = treatment.

Table 8A. Experiment 1. Raw data - ceruloplasmin (abs. at 540 mμ, 3 days/biopsy)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 0.106 | 0.148 | 0.154 | 0.163 | 0.151 |
| | 100 | 0.126 | 0.036 | 0.171 | X | X |
| | 095 | 0.132 | 0.026 | 0.087 | 0.137 | 0.137 |
| | 073 | 0.048 | 0.102 | 0.071 | 0.095 | 0.084 |
| B | 105 | 0.078 | 0.069 | 0.123 | 0.147 | 0.124 |
| | 096 | 0.139 | 0.101 | 0.107 | 0.141 | 0.093 |
| | 004 | 0.123 | 0.103 | 0.129 | X | X |
| | 069 | 0.138 | 0.019 | 0.090 | 0.119 | 0.073 |
| C | 101 | 0.131 | 0.091 | 0.138 | 0.144 | 0.126 |
| | 013 | 0.086 | 0.031 | 0.067 | 0.121 | 0.116 |
| | 081 | 0.018 | 0.025 | 0.136 | 0.166 | 0.127 |
| | 001 | 0.132 | 0.020 | 0.071 | 0.123 | 0.124 |
| D | 106 | 0.130 | 0.158 | 0.132 | 0.200 | 0.165 |
| | 016 | 0.143 | 0.169 | 0.200 | 0.200 | 0.191 |
| | 074 | 0.097 | 0.042 | 0.124 | 0.126 | 0.067 |
| | 083 | 0.114 | 0.170 | X | X | X |

[1] X = missing value.

[2] T = treatment.

Table 9A. Experiment 1. Raw data - TCA-soluble Cu (µg/ml, 3 days after biopsy)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 0.915 | 0.980 | 0.860 | 0.830 | 0.826 |
| | 100 | 0.916 | 0.053 | 0.963 | X | X |
| | 095 | 0.849 | 0.672 | 0.688 | 0.734 | 0.630 |
| | 073 | 0.688 | 0.660 | 0.546 | 0.654 | 0.593 |
| B | 105 | 0.660 | 0.643 | 0.744 | 0.765 | 0.731 |
| | 096 | 0.880 | 0.753 | 0.628 | 0.714 | 0.559 |
| | 004 | 0.810 | 0.765 | 0.778 | X | X |
| | 069 | 0.881 | 0.727 | 0.679 | 0.735 | 0.654 |
| C | 101 | 0.798 | 0.843 | 0.787 | 0.744 | 0.725 |
| | 013 | 0.583 | 0.645 | 0.628 | 0.662 | 0.723 |
| | 081 | 0.800 | 0.880 | 0.886 | 0.791 | 0.838 |
| | 001 | 0.990 | 0.791 | 0.765 | 0.710 | 0.780 |
| D | 106 | 0.813 | 0.944 | 0.890 | 1.114 | 0.776 |
| | 016 | 0.838 | 1.210 | 1.484 | 1.457 | 1.120 |
| | 074 | 0.715 | 0.770 | 0.658 | 0.826 | 0.645 |
| | 083 | 0.733 | 0.840 | X | X | X |

[1] X = missing value.

[2] T = treatment.

Table 10A. Experiment 1. Raw data - TCA-soluble Cu (% of serum Cu, 3 days/biopsy)[1]

| T[2] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| A | 103 | 87.65 | 86.34 | 96.63 | 86.64 | 92.81 |
| | 100 | 97.71 | 88.82 | 97.37 | X | X |
| | 095 | 93.64 | 90.20 | 98.56 | 99.19 | 93.61 |
| | 073 | 97.09 | 94.29 | 80.53 | 92.77 | 96.11 |
| B | 105 | 94.45 | 95.26 | 99.23 | 90.00 | 91.95 |
| | 096 | 77.73 | 82.03 | 86.03 | 94.57 | 84.70 |
| | 004 | 64.81 | 95.03 | 89.94 | X | X |
| | 069 | 98.18 | 88.34 | 80.07 | 90.97 | 95.06 |
| C | 101 | 95.35 | 92.84 | 88.93 | 92.08 | 97.18 |
| | 013 | 99.14 | 93.48 | 85.09 | 95.94 | 93.17 |
| | 081 | 86.75 | 93.41 | 98.44 | 83.26 | 93.53 |
| | 001 | 89.39 | 91.66 | 97.45 | 87.12 | 92.53 |
| D | 106 | 97.29 | 98.03 | 92.90 | 93.61 | 81.00 |
| | 016 | 98.57 | 93.08 | 94.22 | 93.70 | 84.85 |
| | 074 | 97.47 | 98.09 | 72.31 | 93.33 | 86.46 |
| | 083 | 93.45 | 85.71 | X | X | X |

[1] X = missing value.

[2] T = treatment.

Table 11A. Experiment 1. Analysis of variance table for LIVER Cu ($\Delta 1$), mg/kg) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 36865.84 | 12288.62 | 16.74 | ** |
| STEER(TRT) 'Error A' | 12 | 8808.04 | 734.00 | | |
| PERIOD | 3 | 27432.97 | 9144.32 | 26.72 | ** |
| TRTXPERIOD | 9 | 4288.66 | 476.52 | 1.39 | N.S. |
| STEER(TRT X PERIOD) 'Error B' | 30 | 10265.86 | 342.20 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 12A. Experiment 1. Analysis of variance table for SERUM Cu ($\Delta_1$, µg/ml at biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 56745.00 | 18915.00 | 0.50 | N.S. |
| STEER(TRT) 'Error A' | 12 | 455517.74 | 37959.81 | | |
| PERIOD | 3 | 172907.60 | 57635.87 | 3.16 | * |
| TRTXPERIOD | 9 | 112357.92 | 12484.21 | 0.68 | N.S. |
| STEER(TRTX PERIOD) 'Error B' | 30 | 547932.10 | 18264.40 | | |

[1] N.S. = not significant (p>.05)

* = significant at (p<.05)

** = significant at (p<.01)

Table 13A. Experiment 1. Analysis of variance table for CERULOPLASMIN ACTIVITY ($\Delta_1$, abs. at 540 mμ X 100, at biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 10197.93 | 3399.31 | 2.03 | N.S. |
| STEER(TRT) 'Error A' | 12 | 20126.58 | 1677.22 | | |
| PERIOD | 3 | 39745.60 | 13248.53 | 7.97 | ** |
| TRTXPERIOD | 9 | 5963.07 | 662.56 | 0.40 | |
| STEER(TRT X PERIOD) 'Error B' | 30 | 49894.83 | 1663.16 | | |

[1] N.S. = not significant (p>.05)

* = significant at (p<.05)

** = significant at (p<.01)

Table 14A. Experiment 1. Analysis of variance for SERUM Cu ($\Delta_1$, µg/ml, 3 days after biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 0.81934 | 0.27311 | 3.36 | N.S. |
| STEER(TRT) 'Error A' | 12 | 0.97573 | 0.08131 | | |
| PERIOD | 3 | 0.04834 | 0.1612 | 3.39 | * |
| TRTXPERIOD | 9 | 0.08755 | 0.0097 | 2.04 | N.S. |
| STEER(TRT X PERIOD) 'Error B' | 27 | 0.12855 | 0.0048 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 15A. Experiment 1. Analysis of variance table for CERULOPLASMIN ACTIVITY ($\Delta_1$, abs. at 540 mμ, 3 days after biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 0.01632 | 0.00544 | 0.71 | N.S. |
| STEER(TRT) 'Error A' | 12 | 0.09210 | 0.00768 | | |
| PERIOD | 3 | 0.03339 | 0.01113 | 13.09 | ** |
| TRTXPERIOD | 9 | 0.00756 | 0.00084 | 0.98 | N.S. |
| STEER(TRT X PERIOD) 'Error B' | 28 | 0.02385 | 0.00085 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 16A. Experiment 1. Analysis of variance table for TCA-SOLUBLE Cu ($\Delta_1$, as a % of serum Cu, 3 days after biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 1200.61 | 400.20 | 1.80 | N.S. |
| STEER(TRT) 'Error A' | 12 | 2675.11 | 222.93 | | |
| PERIOD | 3 | 50.64 | 16.88 | 0.46 | N.S. |
| TRT X PERIOD | 9 | 365.64 | 40.63 | 1.11 | N.S. |
| STEER(TRT X PERIOD) 'Error B' | 28 | 1028.75 | 36.74 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 17A. Experiment 1. Analysis of variance table for BODY WEIGHT GAIN ($\Delta_2$, kg) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| CTD Total | 12 | 5203.69 | 69.68 | | |
| TRT | 3 | 203.03 | 69.68 | 0.13 | N.S. |
| Error[2] | 9 | 4994.67 | 554.96 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 18A. Experiment 1. Analysis of variance table for LIVER Cu ($\Delta_2$, mg/kg) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| CTD Total | 12 | 21653.72 | | | |
| TRT | 3 | 18239.35 | 6079.78 | 16.03 | ** |
| Error[2] | 9 | 3414.37 | 379.37 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 19A. Experiment 1. Analysis of variance table for SERUM Cu ($\Delta_2$, µg/ml at biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| CTD Total | 12 | 1924.50 | | | |
| TRT | 3 | 345.71 | 115.24 | 0.66 | N.S. |
| Error[2] | 9 | 1578.79 | 175.42 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 20A. Experiment 1. Analysis of variance table for CERULOPLASMIN ACTIVITY ($\Delta_2$, abs. at 540 µm x 100, at biopsy) of beef steers fed different concentrations of Cu and Mo in a 2:1 ratio in the diet for 121 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| CTD Total | 12 | 11988.31 | | | |
| TRT | 3 | 1913.97 | 637.99 | 0.57 | N.S. |
| Error[2] | 9 | 10074.33 | 1119.37 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

# APPENDIX II

Table 21A. Experiment 2. Analysis of variance table for FEED CONSUMPTION (kg/day) of beef steers fed different levels of sulphur in a 4X4 latin square.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 6.63589 | | | |
| Steer | 3 | 0.90819 | 0.30273 | 3.49 | N.S. |
| Treatment | 3 | 0.57677 | 0.19226 | 2.22 | N.S. |
| Period | 3 | 4.63132 | 1.54377 | 17.82 | ** |
| Error | 6 | 0.51979 | 0.08663 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

Table 22A. Experiment 2. Analysis of variance table for LIVER Cu ($\Delta_1$, mg/kg) of beef steers fed different levels of sulphur in a 4X4 latin square.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 6757.63 | | | |
| Steer | 3 | 1116.46 | 372.15 | 6.46 | * |
| Treatment | 3 | 272.79 | 90.93 | 1.58 | N.S. |
| Period | 3 | 5368.38 | 1789.46 | 31.08 | ** |
| Error | 6 | 345.49 | 57.58 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

Table 23A. Experiment 2. Analysis of variance table for SERUM Cu ($\Delta_1$, µg/ml) of beef steers fed different levels of sulphur in a 4X4 latin square.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 0.13852 | | | |
| Steer | 3 | 0.02384 | 0.00795 | 1.00 | N.S. |
| Treatment | 3 | 0.01527 | 0.00509 | 0.64 | N.S. |
| Period | 3 | 0.06150 | 0.02050 | 2.57 | N.S. |
| Error | 6 | 0.04789 | 0.00798 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

Table 24A. Experiment 2. Analysis of variance table for CERULOPLASMIN ACTIVITY ($\Delta_1$, abs. at 540 mμ) of beef steers fed different levels of sulphur in a 4X4 latin square.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 0.02254 | | | |
| Steer | 3 | 0.01160 | 0.00387 | 50.55 | ** |
| Treatment | 3 | 0.00059 | 0.00020 | 2.58 | N.S. |
| Period | 3 | 0.00989 | 0.00330 | 43.08 | ** |
| Error | 6 | 0.00046 | 0.00008 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

Table 25A. Experiment 2. Analysis of variance table for TCA-SOLUBLE Cu ($\Delta_1$, µg/ml) of beef steers fed different levels of sulphur in a 4X4 latin square.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 0.24607 | | | |
| Steer | 3 | 0.05780 | 0.01927 | 2.27 | N.S. |
| Treatment | 3 | 0.03830 | 0.01277 | 1.50 | N.S. |
| Period | 3 | 0.09894 | 0.03298 | 3.88 | N.S. |
| Error | 6 | 0.05103 | 0.00851 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

Table 26A. Experiment 2. Analysis of variance table for TCA-SOLUBLE Cu ($\Delta_1$, % of serum Cu) of beef steers fed different levels of sulphur in a 4X4 latin square.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 987.91 | | | |
| Steer | 3 | 369.30 | 123.10 | 3.08 | N.S. |
| Treatment | 3 | 181.42 | 60.47 | 1.51 | N.S. |
| Period | 3 | 197.46 | 65.82 | 1.65 | N.S. |
| Error | 6 | 239.73 | 39.96 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

# APPENDIX III

Table 27A. Experiment 3. Raw data - Initial body wt. (kg)

| Treatment | Animal | Initial weight | Mean | Std. Dev.[1] |
|---|---|---|---|---|
| 0.2% S | 028 | 273 | 292.50 | 24.25 |
| | 125 | 316 | | |
| | 062 | 270 | | |
| | 005 | 311 | | |
| 0.3% S | 036 | 347 | 311.25 | 37.31 |
| | 031 | 339 | | |
| | 128 | 287 | | |
| | 136 | 272 | | |
| 0.4% S | 010 | 320 | 311.50 | 7.0 |
| | 015 | 304 | | |
| | 048 | 314 | | |
| | 134 | 308 | | |
| 0.5% S | 011 | 334 | 310.00 | 20.70 |
| | 133 | 285 | | |
| | 023 | 317 | | |
| | 008 | 304 | | |

[1]Std. Dev. = standard deviation.

Table 28A. Experiment 3. Raw data - body weight (kg)

| T[1] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| 0.2% S | 028 | 273 | 258 | 304 | 340 | 356 |
| | 125 | 316 | 346 | 374 | 402 | 424 |
| | 062 | 270 | 298 | 324 | 340 | 364 |
| | 005 | 311 | 346 | 372 | 398 | 422 |
| 0.3% S | 036 | 347 | 378 | 392 | 420 | 442 |
| | 031 | 339 | 368 | 394 | 440 | 456 |
| | 128 | 287 | 328 | 342 | 370 | 387 |
| | 136 | 272 | 302 | 328 | 374 | 384 |
| 0.4% S | 010 | 320 | 336 | 364 | 394 | 392 |
| | 015 | 304 | 322 | 368 | 393 | 380 |
| | 048 | 314 | 342 | 374 | 432 | 446 |
| | 134 | 308 | 340 | 356 | 412 | 426 |
| 0.5% S | 011 | 334 | 364 | 388 | 418 | 438 |
| | 133 | 285 | 318 | 330 | 364 | 380 |
| | 023 | 317 | 360 | 368 | 416 | 430 |
| | 008 | 304 | 336 | 356 | 382 | 404 |

[1]T = treatment.

Table 29A. Experiment 3. Raw data - performance

| T[2] | Animal | Final wt. | Gain (kg) | ADG[1] (kg/d) | Dressed (kg) | % of live wt. | Grade |
|---|---|---|---|---|---|---|---|
| 0.2% S | 028 | 356 | 83 | 0.70 | 191.4 | 53.77 | A1 |
| | 125 | 424 | 108 | 0.92 | 223.2 | 52.63 | A2 |
| | 062 | 364 | 94 | 0.80 | 186.0 | 51.09 | A1 |
| | 005 | 422 | 111 | 0.94 | 220.4 | 52.24 | A1 |
| 0.3% S | 036 | 442 | 95 | 0.81 | 221.4 | 50.08 | A1 |
| | 031 | 456 | 117 | 0.99 | 245.8 | 53.91 | A2 |
| | 128 | 387 | 100 | 0.85 | 208.7 | 53.92 | A1 |
| | 136 | 384 | 112 | 0.95 | 207.7 | 54.10 | A1 |
| 0.4% S | 010 | 392 | 72 | 0.61 | 214.1 | 54.62 | A1 |
| | 015 | 380 | 76 | 0.64 | 205.9 | 54.19 | B1 |
| | 048 | 446 | 132 | 1.12 | 237.7 | 53.29 | A1 |
| | 134 | 426 | 86 | 0.73 | 224.1 | 52.60 | A2 |
| 0.5% S | 011 | 438 | 104 | 0.88 | 232.2 | 53.02 | A1 |
| | 133 | 380 | 95 | 0.81 | 204.1 | 53.71 | A1 |
| | 023 | 430 | 113 | 0.96 | 234.1 | 54.43 | A1 |
| | 008 | 404 | 100 | 0.85 | 203.7 | 50.41 | -- |

[1]Based on 118 days.

[2]T = treatment.

Table 30A. Experiment 3. Raw data - liver Cu (ppm, wet basis)

| T[1] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| 0.2% S | 028 | 37.41 | 35.57 | 41.48 | 83.24 | 85.64 |
| | 125 | 15.35 | 55.97 | 95.77 | 94.64 | 105.51 |
| | 062 | 5.88 | 36.55 | 66.82 | 71.94 | 74.78 |
| | 005 | 7.65 | 30.10 | 70.07 | 88.12 | 117.54 |
| 0.3% S | 036 | 14.91 | 32.05 | 40.19 | 45.22 | 55.23 |
| | 031 | 11.47 | 32.37 | 72.25 | 65.96 | 98.62 |
| | 128 | 2.60 | 24.72 | 35.49 | 42.65 | 33.57 |
| | 136 | 5.33 | 30.83 | 70.79 | 72.33 | 97.51 |
| 0.5% S | 010 | 28.02 | 51.22 | 57.58 | 66.00 | 80.64 |
| | 015 | 11.93 | 15.34 | 25.10 | 37.99 | 47.60 |
| | 048 | 18.89 | 29.89 | 36.07 | 46.52 | 80.48 |
| | 134 | 4.46 | 21.90 | 42.34 | 45.70 | 56.86 |
| 0.5% S | 011 | 7.00 | 23.35 | 49.33 | 53.87 | 56.66 |
| | 133 | 15.99 | 31.14 | 86.02 | 94.20 | 115.96 |
| | 023 | 14.74 | 53.68 | 98.75 | 94.91 | 103.41 |
| | 008 | 17.99 | 42.82 | 74.64 | 87.96 | 116.24 |

[1]T = treatment.

Table 31A. Experiment 3. Raw data - serum Cu (µg/ml)

| T[1] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| 0.2% S | 028 | 0.730 | 0.730 | 0.635 | 0.600 | 0.650 |
| | 125 | 0.750 | 0.860 | 0.685 | 0.685 | 0.745 |
| | 062 | 0.810 | 0.800 | 0.760 | 0.740 | 0.725 |
| | 005 | 0.610 | 0.780 | 0.665 | 0.695 | 0.690 |
| 0.3% S | 036 | 0.670 | 0.835 | 0.820 | 0.785 | 0.685 |
| | 031 | 0.675 | 0.920 | 0.675 | 0.500 | 0.580 |
| | 128 | 0.439 | 0.804 | 0.565 | 0.535 | 0.655 |
| | 136 | 0.635 | 0.725 | 0.690 | 0.655 | 0.660 |
| 0.4% S | 010 | 0.755 | 0.820 | 0.740 | 0.695 | 0.590 |
| | 015 | 0.695 | 0.800 | 0.660 | 0.560 | 0.605 |
| | 048 | 0.715 | 0.790 | 0.640 | 0.740 | 0.755 |
| | 134 | 0.625 | 0.690 | 0.970 | 0.670 | 0.625 |
| 0.5% S | 011 | 0.535 | 0.629 | 0.505 | 0.490 | 0.515 |
| | 133 | 0.701 | 0.860 | 0.710 | 0.700 | 0.705 |
| | 023 | 0.716 | 0.705 | 0.590 | 0.555 | 0.630 |
| | 003 | 0.765 | 0.845 | 0.670 | 0.500 | 0.585 |

[1]T = treatment.

Table 32A. Experiment 3. Raw data - ceruloplasmin (abs. at 540 mμ)

| T[1] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| 0.2% S | 028 | 0.127 | 0.095 | 0.096 | 0.080 | 0.076 |
| | 125 | 0.101 | 0.095 | 0.114 | 0.086 | 0.097 |
| | 062 | 0.120 | 0.102 | 0.103 | 0.139 | 0.109 |
| | 005 | 0.076 | 0.088 | 0.077 | 0.089 | 0.097 |
| 0.3% S | 036 | 0.091 | 0.127 | 0.136 | 0.151 | 0.100 |
| | 031 | 0.085 | 0.110 | 0.078 | 0.054 | 0.079 |
| | 128 | 0.045 | 0.096 | 0.076 | 0.067 | 0.087 |
| | 136 | 0.089 | 0.116 | 0.103 | 0.054 | 0.096 |
| 0.4% S | 010 | 0.112 | 0.104 | 0.109 | 0.102 | 0.069 |
| | 015 | 0.107 | 0.110 | 0.089 | 0.077 | 0.049 |
| | 048 | 0.123 | 0.099 | 0.082 | 0.117 | 0.099 |
| | 134 | 0.098 | 0.090 | 0.148 | 0.069 | 0.091 |
| 0.5% S | 011 | 0.076 | 0.071 | 0.080 | 0.065 | 0.056 |
| | 133 | 0.108 | 0.118 | 0.085 | 0.050 | 0.082 |
| | 023 | 0.083 | 0.078 | 0.067 | 0.074 | 0.077 |
| | 008 | 0.113 | 0.098 | 0.103 | 0.032 | 0.059 |

[1]T = treatment.

Table 33A. Experiment 3. Raw data - TCA-soluble Cu (µg/ml)

| T[1] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| 0.2% S | 028 | 0.718 | 0.649 | 0.505 | 0.563 | 0.581 |
| | 125 | 0.668 | 0.662 | 0.654 | 0.637 | 0.626 |
| | 062 | 0.701 | 0.744 | 0.496 | 0.708 | 0.615 |
| | 005 | 0.585 | 0.671 | 0.660 | 0.637 | 0.641 |
| 0.3% S | 036 | 0.662 | 0.761 | 0.613 | 0.766 | 0.629 |
| | 031 | 0.602 | 0.805 | 0.602 | 0.429 | 0.568 |
| | 128 | 0.385 | 0.790 | 0.516 | 0.496 | 0.551 |
| | 136 | 0.615 | 0.701 | 0.604 | 0.591 | 0.572 |
| 0.4% S | 010 | 0.710 | 0.753 | 0.552 | 0.666 | 0.559 |
| | 015 | 0.692 | 0.765 | 0.642 | 0.506 | 0.536 |
| | 048 | 0.675 | 0.787 | 0.530 | 0.728 | 0.643 |
| | 134 | 0.624 | 0.627 | 0.700 | 0.614 | 0.549 |
| 0.5% S | 011 | 0.512 | 0.628 | 0.450 | 0.485 | 0.409 |
| | 133 | 0.673 | 0.825 | 0.624 | 0.539 | 0.585 |
| | 023 | 0.690 | 0.656 | 0.502 | 0.489 | 0.546 |
| | 008 | 0.714 | 0.740 | 0.535 | 0.496 | 0.471 |

[1]T = treatment.

Table 34A. Experiment 3. Raw data - TCA-soluble Cu (% of serum Cu)

| T[1] | Animal | Initial | Period I | II | III | IV |
|---|---|---|---|---|---|---|
| 0.2% S | 028 | 98.36 | 88.90 | 79.53 | 93.26 | 89.38 |
| | 125 | 89.07 | 76.98 | 95.47 | 92.99 | 84.03 |
| | 062 | 86.54 | 93.00 | 65.26 | 95.68 | 84.83 |
| | 003 | 95.90 | 86.03 | 99.25 | 91.65 | 92.90 |
| 0.3% S | 036 | 98.81 | 91.14 | 74.76 | 97.58 | 91.82 |
| | 031 | 89.19 | 87.50 | 89.19 | 85.80 | 97.93 |
| | 128 | 87.70 | 98.26 | 91.33 | 92.71 | 84.12 |
| | 136 | 96.85 | 96.69 | 87.54 | 90.23 | 86.67 |
| 0.4% S | 010 | 94.04 | 91.83 | 74.59 | 95.83 | 94.74 |
| | 015 | 99.57 | 95.63 | 97.27 | 90.36 | 88.60 |
| | 048 | 94.41 | 99.62 | 82.81 | 98.36 | 85.17 |
| | 134 | 99.84 | 90.87 | 77.78 | 91.64 | 87.84 |
| 0.5% S | 011 | 95.70 | 99.84 | 89.11 | 98.98 | 79.42 |
| | 133 | 96.00 | 95.93 | 87.89 | 77.00 | 82.98 |
| | 023 | 96.37 | 93.05 | 85.08 | 88.06 | 86.67 |
| | 008 | 93.33 | 87.57 | 79.85 | 99.20 | 80.51 |

[1]T = treatment.

Table 35A. Experiment 3. Analysis of variance table for LIVER Cu ($\Delta_1$, mg/kg) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 5526.37 | 1842.12 | 1.56 | N.S. |
| STEER(TRT) 'Error A' | 14 | 13869.97 | 990.71 | | |
| PERIOD | 3 | 18271.48 | 6090.49 | 53.70 | ** |
| TRT*PERIOD | 9 | 1678.75 | 186.53 | 1.64 | N.S. |
| STEER(TRT* PERIOD) 'Error B' | 34 | 3855.87 | 113.41 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 36A. Experiment 3. Analysis of table for SERUM Cu ($\Delta_1$, µg/ml) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 0.1691 | 0.05637 | 2.86 | N.S. |
| STEER(TRT) 'Error A' | 14 | 0.2756 | 0.01969 | | |
| PERIOD | 3 | 0.2281 | 0.0760 | 13.76 | ** |
| TRT*PERIOD | 9 | 0.0353 | 0.0039 | 0.71 | N.S. |
| STEER(TRT* PERIOD) 'Error B' | 34 | 0.1878 | 0.0055 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 37A. Experiment 3. Analysis of variance table for CERULOPLASMIN ACTIVITY ($\Delta_1$, abs. at 540 mμ) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 0.0144 | 0.0048 | 3.95 | * |
| STEER(TRT) 'Error A' | 14 | 0.0170 | 0.0012 | | |
| PERIOD | 3 | 0.00357 | 0.0012 | 3.24 | * |
| TRT*PERIOD | 9 | 0.00245 | 0.0027 | 0.74 | N.S. |
| STEER(TRT* PERIOD) 'Error B' | 34 | 0.01251 | 0.0037 | | |

[1] N.S. = not significant (p>.05)

* = significant at (p<.05)

** = significant at (p<.01)

Table 38A. Experiment 3. Analysis of variance table for TCA-SOLUBLE ($\Delta_1$, µg/ml) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 0.1621 | 0.0540 | 2.54 | N.S. |
| STEER(TRT) 'Error A' | 14 | 0.2974 | 0.0212 | | |
| PERIOD | 3 | 0.2563 | 0.0854 | 19.26 | ** |
| TRT*PERIOD | 9 | 0.3980 | 0.0442 | 1.00 | N.S. |
| STEER(TRT* PERIOD) 'Error B' | 34 | 0.1508 | 0.0044 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 39A. Experiment 3. Analysis of variance table for TCA-SOLUBLE Cu ($\Delta_1$, % of serum Cu) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TRT | 3 | 21.94 | 7.31 | 0.16 | N.S. |
| STEER(TRT) 'Error A' | 14 | 630.72 | 45.05 | | |
| PERIOD | 3 | 846.26 | 282.09 | 5.84 | ** |
| TRT*PERIOD | 9 | 297.90 | 33.10 | 0.69 | N.S. |
| STEER(TRT* PERIOD) 'Error B' | 34 | 1642.48 | 48.31 | | |

[1] N.S. = not significant ($p>.05$)

* = significant at ($p<.05$)

** = significant at ($p<.01$)

Table 40A. Experiment 3. Analysis of variance table for FEED CONSUMPTION (kg) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| TOTAL | 15 | 108717 | | | |
| TREATMENT | 3 | 6927 | 2309 | 2.8 | N.S. |
| PERIOD | 3 | 94362 | 31454 | 38.1 | ** |
| ERROR | 9 | 7428 | 825 | | |

[1]N.S. = not significant (p>.05)

* = significant (p<.05)

** = significant (p<.01)

Table 41A.Experiment 3. Analysis of variance table for BODY WEIGHT GAIN ($\Delta_2$, kg) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 3757.75 | | | |
| TRT | 3 | 472.75 | 157.58 | 0.57 | N.S. |
| Error[2] | 12 | 3285.00 | 273.75 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 42A. Experiment 3. Analysis of variance table for LIVER Cu (mg/kg, initial values) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 1252.46 | | | |
| TRT | 3 | 156.23 | 52.08 | 0.57 | N.S. |
| Error[2] | 12 | 1096.23 | 91.35 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 43A. Experiment 3. Analysis of variance table for LIVER Cu ($\Delta_2$, mg/kg) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 9971.58 | | | |
| TRT | 3 | 2859.79 | 953.26 | 1.61 | N.S. |
| Error[2] | 12 | 7111.79 | 592.65 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 44A. Experiment 3. Analysis of variance table for SERUM Cu ($\Delta_2$, µg/ml) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 0.1423 | | | |
| TRT | 3 | 0.0286 | 0.0095 | 1.00 | N.S. |
| Error [2] | 12 | 0.1137 | 0.0095 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 45A. Experiment 3. Analysis of variance table for CERULOPLASMIN ACTIVITY ($\Delta_2$, abs. at 540 μm) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 0.01222 | | | |
| TRT | 3 | 0.00578 | 0.00193 | 3.59 | * |
| Error[2] | 12 | 0.00644 | 0.00054 | | |

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.

Table 46A. Experiment 3. Analysis of variance table for TCA-SOLUBLE Cu ($\Delta_2$, µg/ml) of beef steers fed different levels of sulphur in the diet for 118 days.

| Source | df | SS | MS | F | Conclusion[1] |
|---|---|---|---|---|---|
| Total | 15 | 0.13271 | | | |
| TRT | 3 | 0.05614 | 0.01871 | 2.93 | N.S. |
| Error[2] | 12 | 0.07657 | 0.00638 | | |

ug/ml

[1] N.S. = not significant ($p > .05$)

* = significant ($p < .05$)

** = significant ($p < .01$)

[2] Error mean square was used to calculate the Honestly Significant Difference (HSD) in Tukey's multiple range test at $\alpha = .05$.