The University of Manitoba

The Effect of Molybdenum and Sulfur on Copper Metabolism of Growing Ram Lambs

by

Seyed-Ahmad Moshtaghi-Nia

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree

of Master of Science

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ABSTRACT

The effect of supplemental molybdenum (Mo) and sulfur (S) alone or with copper (Cu) on Cu metabolism and upon the absorption and excretion of Cu and Mo was studied. Twenty-eight ram lambs were allotted to one of four treatment diets on the basis of body weight (34.7±6.60 kg) in a split-plot design. The pelleted diet of alfalfa brome hay:barley (30:64) was fed ad libitum and contained 11.5 mg Cu, 2.8 mg Mo and 1.8 g S/kg DM. The four diets were 1) Control - unsupplemented; 2) 0 Cu - 10 mg Mo + 2 g S/kg; 3) 10 Cu - 10 mg Cu + 10 mg Mo + 2 g S/kg; and 4) 20 Cu - 20 mg Cu + 10 mg Mo + 2 g S/kg. Cu as $CuSO_4 \cdot 5H_2O$, S as Na_2SO_4 and Mo as $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ were added and provided Cu:Mo ratios of 5.0, 0.7, 1.5 and 2.0 for Control, 0 Cu, 10 Cu and 20 Cu diets, respectively. Lambs were kept in confinement with free access to tap water for 16 weeks. Intakes and excretions of Cu and Mo were measured over five days every four weeks on one lamb of average body weight from each treatment diet. Measurement of body weight and samples of liver tissue and blood serum were obtained every four weeks. Samples of liver for Cu, and of serum, feed and feces for Cu and Mo concentrations, were wet ashed and analyzed by atomic absorption spectrophotometry and serum ceruloplasmin (CpOx) was determined by its rate of diamine oxidation. The treatment diets had no significant ($P>.05$) effect on body weight. Liver Cu concentration increased in Control and 20 Cu diets and declined in 0 Cu and 10 Cu diets ($P<.01$). Lambs on the 0 Cu diet had the highest ($P<.01$) concentrations of serum Cu, trichloroacetic acid insoluble Cu (TCAIS Cu) and Mo. Only with the 0 Cu diet were there high correlations

between serum Cu and TCAIS Cu ($r = .87$); serum Cu and serum Mo ($r = .78$); and serum Mo and TCAIS Cu ($r = .87$). CpOx and trichloroacetic acid soluble (TCAS Cu) were higher ($P<.01$) for sheep on the Control diet than for those on the supplemented diets. There were high correlations between serum Cu and TCAS Cu ($r = .92$, $r = .79$ and $r = .84$) for Control, 10 Cu and 20 Cu diets respectively. Serum Cu was correlated with CpOx in Control and 20 Cu ($r = .81$ and $r = .77$) diets respectively. Correlation among the five serum parameters and the one liver parameter were not consistent across diets. This would indicate that interactions among these parameters depended upon diet. Diet by time interaction was significant ($P<.01$) in liver Cu, serum Cu, CpOx, TCAS Cu and TCAIS Cu. The apparent absorption of Cu was lower and more Cu and Mo were excreted in the urine of the 0 Cu lamb than the Control lamb. With increased Cu:Mo ratios in diets (0 Cu lamb vs 10 Cu and 20 Cu lambs), the apparent absorption of Mo was progressively reduced. These results indicate that the interactions among Cu, Mo and S are initiated in the rumen of sheep. It is concluded that, with an adequate dietary level of S and a Cu:Mo ratio of less than 2.0 in the diet, Cu absorption is impaired, liver Cu reserves decrease and the serum Cu increase is associated with high concentrations of serum Mo and TCAIS Cu. These effects were corrected by increasing Cu:Mo ratio in the diet.

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LIST OF ABBREVIATIONS

A	-	absorbance
BW	-	body weight
Cd	-	cadmium
Cp	-	ceruloplasmin
CpOx	-	ceruloplasmin oxidase (EC 1.16.3.1; ferroxidase)
Cu	-	copper
DM	-	dry matter basis
DR-Cu	-	direct reacting copper
Fe	-	iron
g	-	gram
kg	-	kilogram
mg	-	milligram
min	-	minute
ml	-	milliliter
Mo	-	molybdenum
MT	-	metallothionein
S	-	sulfur
SOD	-	superoxide dismutase
TCA	-	trichloroacetic acid
TCAS-Cu	-	trichloroacetic acid soluble copper
TCAIS-Cu	-	trichloroacetic acid insoluble copper
TM	-	thiomolybdate
WB	-	wet weight basis
Zn	-	zinc
Δ	-	delta (change)
µg	-	micrograms

INTRODUCTION

The incidence of naturally occurring copper (Cu) deficiency is almost confined to grazing sheep and cattle. Cu deficiency in the grazing animal can arise from a low dietary intake of Cu or when the herbage has an apparently adequate Cu content, but the presence of factors interferring with the absorption, utilization of storage of Cu. Molybdenum (Mo) in the diet or administered experimentally, interferes with the metabolism of Cu in animals, and the ratio of Cu to Mo in the diet has great significance in the incidence of hypocupremia in sheep and cattle. The interference is not due to a simple chemical reaction of dietary or tissue Cu with Mo but by complex three-way interactions among Cu, Mo and sulfur (S). In ruminants, increased dietary S usually exacerbates the effects of Mo, although Mo retention is decreased (Mason et al. 1978b).

In normal animals Cu in plasma is released from proteins by treatment with trichloroacetic acid (TCA). The oral administration of Mo to sheep increases plasma Cu concentration, but decreases TCA solubility since some of the plasma Cu then precipitates with the plasma protein. The appearance of this new fraction, which also contains Mo is, in sheep, dependent on dietary S (Bremner, 1976). Mason et al. (1978a) showed that this effect of Mo in guinea pigs was also increased by dietary S, but only when S was administered as sulfide. It is thus probable that the higher sensitivity of the ruminant to Mo compared to the non-ruminant is due to rumen reduction of S compounds to sulfide.

Dick et al. (1975) proposed that the sensitivity of ruminants to Mo was due to rumen formation of thiomolybdates $(MoO_{4-n}S_n)^{-2}$, where n = 1 to 4)

by the reaction of molybdate with sulfide which in turn combines with Cu to form the insoluble Cu thiomolybdates, thereby limiting the absorption of dietary Cu. They suggested that the excess thiomolybdate that was not combined with Cu in the rumen was absorbed into the blood stream and combined with tissue Cu, resulting in the appearance of TCA-insoluble Cu in plasma.

There is still not enough information as to the interactions which arise in the gastrointestinal tract or in the blood and whether the antagonistic reactions function by reducing Cu absorption, or by restricting utilization of absorbed Cu. The present study was carried out to determine: a) the metabolism of Cu in the presence of Mo and S; b) the effect of Mo and S on Cu absorption and excretion; c) the ratio of Cu to Mo in the presence of adequate S necessary to maintain Cu balance in sheep.

LITERATURE REVIEW

Copper as an essential element in the body

Copper (Cu) is essential for growth and maintenance of health in all types of farm animals. The actions of Cu at the cellular level generally involve Cu proteins, many of which are enzymes with oxidative functions. Probably no metal ion is more versatile than Cu as a component of specific enzymatic reactions. Cu is a constituent of such enzymes as catalase, tyrosinase, uricase, cytochrome oxidase, monoamine and ascorbic acid oxidase, or is essential for their activity (Hansard, 1983).

Cu is known to facilitate absorption and utilization of Fe, and is necessary for normal bone development. A primary defect of the organic matrix of bone is related to the failure of collagen to undergo cross-linking and maturation. The activity of the Cu metalloenzyme, lysyl-oxidase, decreases severely in Cu deficiency and the mature collagen and elastin are not oxidized (Harris and O'Dell, 1974). Evidence has also been presented for the involvement of Cu in prostaglandin synthesis (Cunnane, 1982) and its role in formation of aortic elastin. The concentrations and activities of many of these enzymes have been related to the specific functional and structural disorders that develop in Cu-deficient animals, as described later in this review.

Copper in blood

Normally Cu appears in serum or plasma in two general forms, bound to macromolecular ligands or to micromolecular ligands. The major

macromolecular ligand, ceruloplasmin (Cp), binds approximately 90% of circulating Cu. The other macromolecular Cu ligand is albumin which binds approximately 9% of the plasma Cu. This Cu is in equilibrium with Cu bound to micromolecular ligands, mainly the amino acids histidine and cysteine, which represent about 1% of the plasma Cu (Henkin, 1974). Cu in its cupric form is relatively loosely bound to albumin and reacts with diethyldithiocarbamate even though the pH remains above 7. Albumin-bound Cu, therefore, is called the "direct-reacting Cu" (DR-Cu) of plasma. Conversely Cu is very tightly bound to Cp and thereby determines the conformation of the molecule. The Cp-bound Cu is referred to as the "indirect-reacting Cu" of plasma since it will not react with sodium diethyldithiocarbamate unless rendered ionic by acid cleavage of the protein-to-Cu linkage (Aspin and Sasskortsak, 1981).

Cp is a blue Cu protein found in the 2-globulin fraction of plasma. It is a single-chain glycoprotein of molecular weight 132000 and contains six Cu atoms per molecule. Its many functions may be related to the heterogenous nature of these six Cu atoms and to the various catalytic activities which they provide (Frieden, 1980). Cp (ferroxidase) mobilizes Fe into the plasma from Fe storage cells in the liver and functions to oxidize ferrous to ferric iron that then becomes bound to transferrin. An equally important function is that Cp, serves as a major transport vehicle for Cu. Thus the Cu atoms of Cp are a prerequisite for Cu utilization. A possible third role of Cp is as a contributor to regulation of the balance of biogenic amines through its oxidase action on epinephrine and the hydroxyindole series.

The rate of synthesis of Cp varies among species as Marcilese et al.

(1976) have shown a higher rate of Cp production in cattle than in sheep, although the half-time of Cp in the circulation of cattle seems to be shorter than in sheep. These authors concluded that these differences in kinetics of Cp between cattle and sheep could explain the higher susceptibility of cattle to the effects of dietary Mo and S. Calabrese et al. (1983) showed that sheep Cp differs from bovine Cp because of its higher specific enzyme activity.

Dramatic changes in Cp levels occur during pregnancy in sheep. Cp levels fall in early pregnancy and remain low during the last half of pregnancy, but rise at the time of parturition to reach the highest levels one week after lambing. In the lambs Cp levels are low at birth and at 24 hr after birth, but by one week after birth, the values are well within the range for normal healthy adults (Butler, 1963; McCosker, 1968a; Howell et al., 1968; Bingley and Dufty, 1969; Hidiroglou and Knipfel, 1981). Genetic variation in Cp levels has also been demonstrated as Wiener et al. (1974) found that Welsh Mountain sheep had higher levels of Cp than Scottish Blackface sheep and crosses of the two breeds of sheep had intermediate Cp levels.

Approximately 60% of the total Cu in red blood cells is associated with the enzyme superoxide dismutase (SOD), formerly known as erythrocuprein which contains 0.34% Cu. SOD is a metalloenzyme that catalytically scavenges the superoxide radical and is essential for the aerobic survival of all forms of life. SOD contains two Cu and two Zn atoms and has a molecular weight of about 32000. The Cu in SOD participates in the catalytic activity of the enzyme, but the Zn plays only a structural

role. SOD provides protection against oxygen toxicity, against compounds that cause exacerbation of oxygen toxicity, against ionizing radiation, and also against damaging sequelae of prolonged inflammation (Hassan, 1980).

The second fraction of erythrocyte Cu is contained within a freely dialyzable component that is designated the "labile pool". This component contains Cu complexed with amino acids and it is probably necessary to insure an adequate supply of Cu to maintain the activity of SOD (Evans, 1973 and Underwood, 1977). The total Cu content of erythrocytes remains constant in spite of the Cu status of the animal and parenteral injection of an excessive amount of Cu does not produce a significant increase in erythrocyte Cu content (Evans, 1973). Thus, although the Cu in erythrocytes is circulating, this fraction of blood Cu is not involved in transporting Cu. Variation attributable to breed of sire in sheep have also significant effects on SOD activity (Woolliams et al., 1983).

The normal range of Cu concentration in the blood or plasma of healthy ruminant animals is 0.80-1.20 µg/ml (Underwood, 1977; Church et al., 1979; Grace, 1983). Several factors including breed, pregnancy, age and diet alter the blood Cu level in animals. Significant breed differences have also been demonstrated. For example, Finnish Landrace sheep have markedly lower plasma Cu concentrations than Merinos (Hayter and Wiener, 1973).

Butler (1963) found that ewes maintained on a constant diet had declining whole blood, plasma, Cp and erythrocyte Cu levels during pregnancy. The blood Cu and Cp levels increased at parturition and reached the highest levels one week after lambing. The pregnant ewe

appears to be poorly equipped to protect her lamb against effects of dietary Cu deficiency. Causes of the reduction in the Cu content of the blood during pregnancy may be physiological adjustments such as increased maternal blood volume as well as the requirement of the developing fetus (Hidiroglou and Knipfel, 1981). In lambs, blood Cu and Cp levels were low at birth and 24 hr later but were within the normal adult range by one week of age (Howell and Edington, 1968). In the bovine, whole blood and plasma Cu levels are lower and erythrocyte Cu levels higher in newborn calves than in their mothers (Bingley, 1974).

Subnormal levels of dietary Cu are reflected in subnormal blood Cu concentrations in sheep and cattle. Cu levels of less than 0.80, 0.70 and 0.60 mg/ℓ for whole blood, plasma and serum Cu respectively have been defined as indicative of a Cu deficiency (Suttle, 1983). Ingestion of high dietary levels of elements such as Zn, Cd and Fe depress absorption of Cu and can reduce plasma Cu concentration. The effects of Mo and S depend on the status of the animal with respect to these nutrients and Cu. Prolonged high intakes of Mo and S cause striking changes in the concentrations of Cu in the blood and in its distribution among the blood components in sheep (Bingley, 1974). Hypercupremia occurs as a consequence of extremely high dietary Cu intakes. During the terminal stages of Cu poisoning, i.e. within 24-48 hr of the hemolytic crisis in sheep, blood concentrations as high as 10-14 μg Cu/ml have been reported (Underwood, 1977).

Copper in liver

The slow development of Cu deficiency signs in animals receiving low-Cu diets indicates that many species possess appreciable stores of

Cu. The main storage organ for Cu is the liver, although appreciable amounts of Cu accumulate in other tissues. Liver Cu concentrations vary, with the species and age of animal, the chemical composition of the diet, and in various disease conditions.

Sheep and cattle have liver Cu concentrations with a normal range of 100-400 mg/kg dry matter (DM). It seems probable that sheep and cattle are superior to rats in capacity to bind Cu in the liver and blood Cu levels do not rise in these species with increased Cu intakes as in rats, except at a very high dietary intake (Underwood, 1977). Liver Cu concentrations are lower in newborn sheep than in adult sheep and in cattle, they change little from birth to old age (McCosker, 1968a). The concentration of Cu in liver is very sensitive to low Cu intakes and liver Cu provides a useful aid in the diagnosis of Cu deficiency. The minimum liver Cu level necessary to maintain a normal plasma Cu level has been estimated to be approximately 40 mg/kg DM in cattle (Claypool et al., 1975). Dick (1954) studied liver Cu storage in sheep ingesting graded increments of Cu from 3.6 to 33.6 mg/day for 177 days. The liver Cu concentrations increased steadily from 562 mg/kg DM at the lowest to 2340 mg/kg DM at the highest Cu intake.

Liver Cu is affected by other dietary factors that influence Cu retention in the body through their effect on Cu absorption, excretion, or both. The storage of Cu in the liver of sheep and cattle can be reduced significantly by an increase in dietary Mo, provided that dietary S intakes are adequate.

There are three different proteins in the liver which can store Cu,

namely, hepatocuprein (SOD), metallothionein and mitochondrocuprein. Although SOD can account for 20-50% of the total hepatic Cu, this occurs only when Cu concentrations in the liver are low. As liver Cu increases, the relative contribution made by this protein decreases (Bremner, 1980).

Copper metabolism in the ruminant animal

Absorption

Although low dietary intake of Cu is a frequent cause of a deficiency disease, a deficiency can often arise when the dietary concentration of Cu would normally be considered adequate. Such situations arise when dietary intake of Cu, the physiological state or age of the animal, dietary composition or animal genetic variables influence either absorption or utilization of Cu. Reliable estimation of the effects of such factors on Cu metabolism is often the most difficult problem encountered when attempting to determine the adequacy or otherwise of dietary supply.

One of the major determinants of dietary Cu utilization is its chemical form in the diet and in the intestinal contents at the site of absorption. Lassiter and Bell (1960) studied the availability to sheep of various compounds labelled with ^{64}Cu. The Cu in copper oxides is less available than that in water-soluble salts or the carbonate. Later Chapman and Bell (1963) tested the uptake of ^{64}Cu from several inorganic compounds by beef cattle. The relative appearance of ^{64}Cu in the blood of cattle was in the following order: $CuCO_3 > Cu(NO_3)_2 > CuSO_4 > CuCl_2 > Cu_2O >$ CuO(Powder)>CuO(needles)>Cu(wire).

In studies with sheep marked differences have been reported for the availability of Cu from herbages and practical type diets. Thus the apparent availability of Cu from fresh perennial ryegrass and white clover was 30 and 34% respectively whereas it was only 9% from red clover (Grace, 1975). In another experiment in which the relative proportions of ground straw, ground barley and dried grass meal in the diets were varied, the availability of the dietary Cu ranged from +6 to -15% (Stevenson and Unsworth, 1978; Bremner and Davies, 1980).

Beyond the evidence that virtually all of the water soluble Cu of herbage is present as a range of Cu complexes stable down to pH 2, little is known either of the forms in which Cu occurs in the diet or of the extent to which these forms persist during the early stages of digestion in the rumen, abomasum or stomach (Bremner, 1970). The relatively ineffective absorption of Cu from the intestines may be related to the finding that the proportional solubility of Cu is much lower in the abomasum, despite its low pH, than in other regions of the digestive tract. Such decreased solubility of Cu can be reproduced experimentally merely by acidification of rumen liquor to simulate the pH of abomasal contents (Bremner, 1970 and Mills, 1980b).

An appreciable proportion of ingested Cu is incorporated into the cell walls of rumen organisms. It appears that much of this cell wall Cu may subsequently escape digestion and absorption. The extent to which variations in rumen bacterial or protozoal activity influence this loss has recently been assessed by Ivan and Veira (1982). They carried out an experiment to measure the soluble proportions of Cu in the rumen fluid

and duodenal digesta, and the flow into the small intestine in defaunated and faunated sheep. It was concluded the ciliate protozoa do not have a critical role in Cu metabolism in the rumen of sheep.

The absorption of Cu has also been related to the nutritional quality of the ration (Lamand, 1978). For example, additional urea, at the rate of 6 g/kg fresh weight of corn silage fed to sheep, increased the apparent absorption of Cu from 3.4% to 18.5% (Ivan et al., 1983). It is possible that differences in the fermentation processes, in the corn silages and in the rumen, resulted in a greater biological availability of Cu in the urea supplemented silage.

Normally ruminants absorb less than 10% of dietary Cu but efficiency of absorption may be influenced by other dietary constituents. The most striking examples concern the influence of S on the absorption of Cu and Mo. With semi-purified diets the effects on Cu are summarized by the equation:

$$\text{Log } A_{Cu} = -1.113 - 0.0714xS - 0.0187xSxMo$$

where S and Mo are dietary concentrations in g and mg/kg DM respectively and A_{Cu} is the absorption coefficient for dietary Cu (Suttle, 1979). From this equation it can be calculated that A_{Cu} will decrease to about 1/3 (from 0.06 to 0.02) as S and Mo increase within normal limits.

The solubility of Cu and thus the facility with which it can be absorbed is markedly reduced by the presence of sulfide in the gastrointestinal tract whether this originates from the diet or, as in ruminants, is generated by the reduction of dietary sulfate or the degradation of sulfur amino acids (Bird, 1970). Suttle (1974a) measured the effect of

S on the availability of Cu from a semi-purified diet with sheep. Increasing the S content of the diet from 1 to 4 g/kg reduced the availability of the Cu by about 40%. The mechanism by which this occurred involved generation of S^{2-} in the rumen and the formation of insoluble CuS, which is known to be a relatively nonavailable form of Cu. Presence of Mo as molybdate in the intestinal lumen has little or no effect on Cu absorption in any species so far investigated. In contrast the tetrathiomolybdate ion (MoS_4^{2-}) which, it has been suggested may be formed in the rumen from the reaction of molybdate with sulfide and may be a key intermediate in the Mo/Cu antagonism, strongly inhibits Cu absorption (Dicks et al., 1975; Mills, 1980a).

The apparent availability of Cu is high for milk-fed animals compared with more mature ruminating animals. In a study with lambs the efficiency of Cu absorption was 71% at 28 days before weaning, 47% at 14 days prior to weaning but only 8-10% after weaning (Suttle, 1975). In similar experiments with calves fitted with duodenal and ileal reentrant cannulae, absorption from mouth to duodenum, mouth to ileum, and mouth to anus in the milk-fed animal was 10, 59 and 68% respectively, compared with 10, 19 and 27%, after weaning. This indicates that the decline is absorption from the small intestine was mainly responsible for the decrease in net Cu absorption after weaning (Bremner and Davies, 1980).

The mechanisms which regulate Cu absorption involve metal-binding components and the inhibition of Cu absorption brought about by various metals, results from competition for protein metal-binding sites. Evans

and Haln (1974) found that orally administered Cu becomes associated with a variety of metal-binding ligands and macromolecules in the intestine of the rat. In the intestinal lumen, Cu was complexed with a protein similar to metallothionein (MT), which could conceivably be involved in transporting Cu across the intestinal epithelium.

It has been suggested that Cu absorption involves two distinct steps: the uptake of Cu from the intestinal lumen into the mucosal cells and the subsequent transfer of this Cu into portal circulation. High concentrations of Zn interfere with the absorption of Cu, indicating competition between these ions for the same binding sites (Van Campen and Scaife, 1967). Fischer et al. (1983) found with a low Zn diet, that the limited amount of MT present was saturated, and the excess Cu was bound to the high-molecular-weight protein fraction (HMWPF). With larger intakes of Zn, MT synthesis was induced, and the larger amount of this protein was not as readily saturated, resulting in less Cu binding to the HMWPF and more to MT. They suggested that Zn interferes with Cu absorption by inducing MT, which sequesters Cu in the mucosal cells, making it unavailable for serosal transfer. The Cu bound to the HMWPF was available for transfer.

Transport of copper

Most of the Cu in plasma is generally present in the form of Cp, an α-globulin, which binds Cu atoms in nonexchangeable forms. However, the principal transport forms of Cu are its loosely bound complexes with albumin and, to a lesser extent, with selected amino acids such as

histidine, threonine and glutamine. These complexes are probably in equilibrium with one another, and altogether they usually account for 5-10% of the total plasma Cu (Bremner, 1980). The absorbed Cu is rapidly incorporated into complexes with albumin and with amino acids in plasma and are taken up by the liver and other tissues.

The release of Cu from plasma albumin is affected in ruminant animals receiving diets with a high Mo and S content. Such animals have increased plasma Cu concentrations, which probably arise from a decreased rate of clearance of Cu from the plasma (Smith et al., 1968). This, in turn, appears to be a consequence of the formation in the rumen of thiomolybdate which, when absorbed, may enhance the affinity of albumin for Cu (Mills and Bremner, 1980).

The Cu reaching the liver, the main storage organ of the body for Cu and a key organ in the metabolism of this element, is incorporated into the mitochondria, microsomes, nuclei, and soluble fraction of the parenchymal cells in proportions which vary with the age, strain, and the Cu status of the animal (Underwood, 1977). Hepatic Cu is then (a) incorporated into Cp and released into plasma or (b) released into bile or (c) stored temporarily in the liver.

Excretion

In all species studied, a high proportion of ingested Cu appears in the feces. Most of this is normally unabsorbed dietary Cu, but active excretion occurs via the bile. One function of the hepatic cells is to prepare Cu, which is in excess of the body's requirement, for excretion

in the bile. Sheep and other ruminants excrete Cu in bile at very low rates that are approximately equal to the rates of Cu excretion in urine (Sali and Ramback, 1978). The concentration of Cu in the liver does not influence the excretion of Cu in bile. Lysosomes are important in the excretion of Cu in bile. The defect in Cu homeostasis in sheep may be the inability of lysosomes to sequester and excrete the Cu in the liver. This could be due to a difference in lysosomal properties or lysosomal numbers (Hubbs and Oehme, 1982).

Although Cu is rapidly taken up by kidneys after its absorption from the gut or after intravenous injection of the metal, urinary concentrations of Cu are usually quite low, amounting to only 1 to 2% of the total Cu excreted by sheep fed purified diets (Smith et al., 1968) or by cattle fed grass or alfalfa hay (Lesperance and Bohman, 1963). However, increased renal concentrations have been reported in Cu-poisoned sheep (Bremner, 1979; Gooneratne et al., 1981b). In these animals, the additional Cu was present principally as MT in kidney or in an uncharacterized protein with the same molecular weight as MT (Bremner, 1980).

Marcilese et al. (1970) found that the addition of Mo and S to the diet of sheep caused a greater accumulation of Cu in the kidney and increased filtration in the kidney may have contributed to the increased urinary Cu when Mo and S were added to the diet.

Copper, molybdenum and sulfur interactions

The interactions among dietary Cu, Mo and S, are most widely known and thoroughly researched. The interactions are complex and operate both in the digestive tract and systemically. The availability of

dietary Cu is reduced by increasing Mo concentrations in the presence of an adequate level of S. The clinical appearance of Cu toxicity and deficiency were originally associated with very low and very high levels of dietary Mo (respectively) and these observations initially prompted the many investigations that have been conducted (Underwood, 1977).

Sheep have frequently been used in Cu-Mo studies although they are less sensitive than cattle to Mo excess. Liver Cu storage was reduced in sheep receiving 9 mg Cu/day when Mo intake was raised from 5 to 20 mg/day (Dick, 1954). Mo was found to reduce Cu retention, but this effect was only observed when the diet also contained a sufficient quantity of inorganic sulfate (Dick, 1954; Kline et al., 1971; Ross, 1970 and Weber et al., 1983). The direct antagonism between Cu and sulfate may be due to the formation of cupric sulfide. Lambs fed purified soybean protein did not retain or store as much Cu as did those fed urea; the addition of sulfur as sulfate decreased the retention and liver storage of Cu (Goodrich and Tillman, 1966). Merry et al. (1983) have suggested that diets in South Australia containing appreciable quantities of cruciferous species, which have low Cu but high sulfur concentrations, could induce Cu deficiency in ruminants.

In many studies, however, supplementation of either Mo or S alone did not affect Cu status (Marcilese et al., 1969 and Suttle, 1974b). Whether or not interactions are observed depends greatly upon the basal content of Mo and S and upon the ratio between them. Simpson et al. (1982) found that increasing dietary S from 1.8 to 3.2 g/kg DM had no influence upon Cu retention of growing cattle unless Mo was also increased. As far as can be ascertained, the Mo content of their basal

diet (<0.1 mg/kg DM) was lower than those reported in similar studies.

With respect to cattle, Vanderveen and Keener (1964) reported that heifers which received diets containing from 5 to 50 ppm Mo and no added sulfate did not develop any symptoms of Mo toxicity, but Cu levels in the liver and blood serum was lowered. Heifers receiving 50 ppm Mo and 0.3% sulfate sulfur developed alopecia and achromatrichia. Cu added to the diet of heifers which developed achromatrichia and alopecia completely corrected these conditions. Humphries et al. (1983) found that liver, plasma Cu concentrations and plasma Cp activities decreased greatly and rapidly in all calves given Mo supplement (5 mg/kg) in a diet containing 4 mg Cu/kg and 2.8 g S/kg DM.

From the experimental results it may be concluded that high Mo and sulfate intakes impair the metabolic efficiency of Cu utilization and therefore induce Cu deficiency. From studies by Marcilese et al. (1969) with labelled Cu, it was evident that Mo and sulfate supplementation decreases the incorporation of Cu into the liver and the synthesis of Cp. Accordingly, high levels of Cu supplementation are required to counteract these effects of Mo and sulfate.

In considering the aspect of chemical parameters in Cu, Mo and S interactions, the elements in the periodic table tend to react in a chemical manner similar to other elements in the same family group (vertical column) due to their similar outer electronic configurations (Huisingh and Matrone, 1976). Matrone (1974) proposed that ions with similar electron structure, geometric configuration, and coordination number would interact antagonistically in biological systems. For

example, Cu^{2+}, Zn^{2+} and Cd^{2+} all have ten electrons in the outer d orbitals, an sp^3 or tetrahedral geometric configuration, and tend to form coordination complexes with four ligands. These elements interact antagonistically in animals, presumably because of their chemical similarities. Mo can exist in several oxidation states, it is most stable in the +6 oxidation state where it is usually bound to four oxygens and exists as the oxy-anion molybdate (MoO_4^{2-}). Huisingh and Matrone (1976) predicted from the chemical parameter concept, that molybdate would be biologically antagonistic to other oxy-anions in the same group or similar group, for example, chromate (CrO_4^{2-}) and sulfate (SO_4^{2-}). Since sulfate is normally present in animal diets at a much higher concentration than the other oxy-anions, the interaction between sulfate and molybdate is of major significance. Biological interactions of these oxy-anions with several cations including Cu and Fe have been observed.

Dick (1954) showed that added dietary inorganic sulfate could decrease Cu storage. Spais et al. (1968) demonstrated that the rumen contents of animals fed diets containing high levels of inorganic sulfur compounds also contained high levels of sulfide. Ruminants, in contrast to monogastrics, are able to reduce sulfate to sulfide to a major extent in the rumen (Suttle, 1974a) because of the action of Desulfovibrio bacteria present in the ruminal contents (Huisingh et al., 1974). The addition of Cu to washed cultures of these microorganisms from the ovine rumen depressed the reduction of sulfate to sulfide (Nikolic et al., 1983); Mo addition however, did so very effectively even at low concentration. When Cu and Mo were added together to the culture medium, the effect of the latter element was diminished (Huisingh et al., 1974 and

Kirchgessner et al., 1979).

Suttle (1974a) studied the responses of initially hypocupremic ewes to the repletion with Cu-supplemented diets containing supplements of organic S, as methionine, and of inorganic S as Na_2SO_4. The S supplement had similar effects. Responses in plasma Cu were reduced when S was increased. He concluded that the variation in the form of dietary S within normal range for herbage exerts an independent effect on Cu metabolism, possibly through the formation of insoluble CuS, because the addition of CuS (5 mg Cu/kg) produced no response in plasma Cu but the same amount of Cu, given as $CuSO_4$, increased plasma Cu.

Ivan and Veira (1981) reported that the solubility of Cu was proportionally decreased in both rumen and abomasal digesta with increased dietary protein. They suggested that high levels of very soluble or degradable protein in pasture is responsible for formation of insoluble CuS during rumen fermentation, resulting in lower solubility and absorption of Cu. Huisingh et al. (1975) attributed similar results to the fact that sulfide is liberated from sulfur-containing amino acids in the rumen. While Mo inhibits the production of sulfide from sulfate, it activates sulfide liberation from sulfur-containing amino acids, so that the total sulfide production in the rumen depends upon the ratio of sulfate and S from organic sources in the diet. These concepts are also discussed by Gawthorne and Nader (1976) who assumed, in addition, that the apparent absorption of sulfide from the rumen was decreased when Mo was infused daily (50 mg molybdate/day), since they found that the sulfide contents in the ruminal fluid rose overall, while the de novo synthesis of sulfide was significantly lowered by Mo supplementation.

In considering the effect of Mo on Cu metabolism, Dowdy and Matrone (1968) observed that $CuSO_4$ and Na_2MoO_4 form a complex which precipitates in a near neutral solution. The ratio of Cu to Mo in this complex was 4:3. They hypothesized that Mo complexes with Cu and that Cu bound in this state is apparently absorbed, but the Cu seems to be systemically unavailable from this complex.

The addition of high concentrations of Mo and S to the diet of sheep resulted in increased plasma Cu, direct reacting Cu, decreased liver Cu concentration and increased Cu and Mo concentration in kidneys (Bingley, 1974; Smith and Wright, 1975a; Marcilese et al., 1970; Van Ryssen and Stielau, 1981). In this situation, a fraction of plasma Cu which is insoluble in trichloroacetic acid (TCA) was present and it has been shown that this fraction also contains Mo. Smith and Wright (1975b) estimated that atomic ratio of Cu:Mo in this fraction was 1.7. It is suggested that the formation of such a stable Cu-Mo-protein compound may explain the observed low tissue uptake of Cu in the presence of high plasma total and direct-reacting Cu concentrations.

Smith and Wright (1976) studied the effect of dietary Mo (17 ppm) and S (5.3 g SO_4/kg) on the metabolism of injected ^{64}Cu in sheep. They found that the inclusion of Mo in the diet caused a retention of ^{64}Cu in whole plasma and the existence of 30% ^{64}Cu in a fraction, insoluble in TCA, which did not exist in the control animals. In a similar study by Suttle (1974b), but with the diet providing 1.7 g S/kg the addition of Mo to the diet did not reduce the effectiveness of injectable Cu. He suggested that the primary site for Cu x Mo interaction is located in the gut, possibly through the formation of a Cu-complex which is absorbed

but which remains biologically unavailable to the tissues and which is excreted by the kidneys.

In a review of Cu, Mo and S interactions, Goodrich et al. (1978) summarized the effects of dietary Mo and S on Cu metabolism as follows. High levels of dietary S result in lowered blood Cu and Mo, lowered liver Cu levels and increased fecal excretion of Mo. High levels of dietary Mo inhibit the storage of Cu. The combination of high dietary levels of S and Mo appear to have a greater effect on the Cu metabolism of ruminants than either element singly, causing decreased blood and liver Cu, increased fecal and urinary Cu losses, reduced absorption of dietary Cu and depletion of tissue Cu.

Huisingh and Matrone (1976) proposed a model to rationalize the Cu, Mo and S interaction in ruminant animals. Cu can become unavailable in the rumen because of either: (1) formation of cupric molybdate or (2) formation of cupric sulfide. The latter is probably more significant, and the availability of Cu is thus greatly dependent upon the pool of ruminal sulfide. Molybdate can affect the pool of sulfide because:

a) if the diet contains sulfate as the major sulfur source, then molybdate decreases the concentration of sulfide by inhibiting sulfate reduction. In this case molybdate alleviates Cu deficiency symptoms.

b) if the diet contains sulfur amino acids (in protein) as the major source of sulfur, then molybdate will increase the concentration of sulfide formed from methionine and possibly the other sulfur containing amino acids. In this case, molybdate aggravates the Cu deficiency symptoms.

In the intestinal tract, a significant interaction is the competition between sulfate and molybdate for absorption via a proposed common carrier. A similar absorption mechanism exists in the kidney at the site of tubular reabsorption and would explain the increased excretion of molybdate in the presence of sulfate.

Huisingh and Matrone found that cupric molybdate does not exist as such in the serum, and that Cu from cupric molybdate becomes bound to serum proteins but molybdate appears to exist as the unbound anion.

Sulfide produced in the rumen, either from sulfate or S-amino acids, is transported to the liver where it is detoxified by oxidation to sulfate, which may either be utilized by first being activated to APS and consequently to 3'-phosphoadenosine-5'phosphosulfate (PAPS), or it may be excreted via the urine as sulfate.

It has been suggested that many aspects of the Cu, Mo and S interaction can be related to the formation in the rumen of thiomolybdate (TM) derivatives (Dick et al., 1975; Mills et al., 1978; Suttle, 1980). The coordinating ligand is postulated to have the general formula $(MoO_{4-n}S_n)^{-2}$ where n = 1 to 4. Dick et al. (1975) and Mills (1980a) attempted to formulate a model which described the mechanism involved in the inhibitory action of dietary Mo and S upon Cu utilization by ruminants. It was suggested that there are three essential steps: 1) Reduction, in the rumen, of sulfate to sulfide; 2) the reaction, at relatively neutral pH, of sulfide with molybdate in the rumen to produce TM, and 3) the reaction of the TM with Cu to give very insoluble Cu thiomolybdate (CuTM). It has been suggested that such insoluble CuTM is

not available for absorption but that TM which is not combined with Cu is absorbed. Absorbed TM combines with mobilized tissue Cu, giving rise to elevated blood Cu values, the increment being insoluble in TCA and its magnitude, for any given Cu intake, being related to Mo intake (Dick et al., 1975; Mason et al., 1978b; Mills and Bremner, 1979).

A wide range of dietary Mo (15 - 60 mg/kg) content has been shown to deplete liver Cu reserves, increase plasma Cu, and ultimately provoke clinical Cu deficiency in ruminants maintained on diets adequate in S content (Lamand et al., 1980; Ishida et al., 1982). The rise in plasma Cu is associated with the appearance in plasma of protein fractions that do not release their Cu on treatment with TCA (Mason et al., 1978). The administration of TM in the diet (Suttle and Field, 1983) into the duodenum (Mason et al., 1980) or by intravenous injection (Gordon and Hill, 1982; Gooneratne et al., 1981b) caused increased plasma Cu and TCA-insoluble Cu and decreased liver Cu reserves. Similarities in the physiological effects of TM and $MoO_4^{2-} + SO_4^{2-}$ in ruminants suggest strongly that TM is formed at an early stage of the $MoO_4^{2-} \times SO_4^{2-}$ interaction in the S^{2-} rich environment of the rumen.

In summary, at high dietary Mo and S concentrations, sufficient TM forms in the rumen and complexes with Cu to impair Cu absorption. In the event of relative TM excess, more absorption of TM occurs and it will have systemic effects on Cu metabolism in ruminants.

Copper toxicity in the ruminant animal

Although copper (Cu) is an essential element required for a wide

range of metabolic processes in the body, excessive intake of this metal can have serious effects in animals. Depending on the species involved, growth and feed intake may be reduced, anemia can develop, and considerable damage may occur to the liver, kidneys, brain and muscle, often resulting in death.

Occurrence of copper toxicity

In all animals the continued ingestion of Cu in excess of requirements leads to accumulation in the tissues, especially in the liver. The capacity for hepatic Cu storage varies greatly among species, and differences among species in tolerance to high-Cu intakes are also great.

It has been known for many years that sheep are more susceptible to Cu toxicity than cattle. Young lambs develop Cu toxicity at relatively low Cu intakes, especially when they are receiving milk-based diets (Bundza et al., 1982). Older calves and adult cattle are much more tolerant of Cu, and the few cases of Cu poisoning which have been reported have involved the supplementation of large amounts of this element (Blakley et al., 1982). There are no reports of Cu toxicity occurring naturally in goats, although a recent study (Wasfi and Adam, 1976) has shown that this condition can be induced at relatively high levels of Cu intake.

In sheep, both acute and chronic Cu poisoning occur under field conditions. Acute Cu poisoning usually occurs because of the accidental administration of large quantities of soluble Cu salts, but chronic Cu poisoning is mainly a condition which occurs in sheep on natural grazing

under the following sets of conditions:

1) when the Cu content of the soils and pastures are abnormally high. These pastures are growing on the cupriferous soils of the area, and the Cu is consumed by the animals from soil and dust. Some plants growing on these soils contain as much as 50 - 60 mg Cu/kg DM (Underwood, 1977).

2) when Cu levels are normal but the molybdenum (Mo) levels are very low. The condition is usually seasonal in occurrence, appears less in Merinos than in British breeds or crosses (Church et al., 1979), occurs only on the more acid soils of the region, and is favored by dominance of the pastures with the clover Trifolium subterraneum. This plant generally contains 10 - 15 mg Cu/kg and extremely low Mo levels that rarely exceed 0.1 - 0.2 mg Mo/kg (Underwood, 1981). Mo is a potent antagonist of Cu metabolism in ruminants (Dick, 1954), and it was suggested that the low Mo intake of the sheep permits excessive hepatic accumulation of Cu.

3) in association with liver damage due to poisoning by the plant Heliotropium erropaeum. This contains hepatotoxic alkaloids which apparently induce changes in the size and life span of liver cells and causes substantial increases in liver Cu concentration and susceptibility to Cu poisoning. This disease is known as toxemic jaundice (Bremner, 1979).

Losses of sheep from chronic Cu poisoning have been reported from the ingestion of herbage in orchards and vineyards previously sprayed with Cu compounds and from pastures sprayed with Cu-sulfate as a molluscicide. It may also occur on dry feed and at pasture from continued

free choice consumption of mineral salt mixtures containing recommended amounts of Cu (Underwood, 1977).

The use of high dietary concentration of Cu in rations for pigs and poultry is now widespread. Excreta from these animals contains relatively high concentrations of Cu (Suttle et al., 1978). The recycling of animal wastes as fertilizers or as dietary supplements therefore constitutes a potential Cu toxicity hazard to susceptible species such as sheep. Feeding high Cu broiler litter (125 mg Cu/kg) to ewes increased the liver Cu concentration to 1425 mg Cu/kg DM (Olson et al., 1982). These authors indicated that Cu in the liver cannot be depleted from ovine liver by removing the litter from the diet. High concentrations of Cu have been reported for treated pastures. Most of this Cu is present as surface contamination as there is relatively little uptake of Cu by plants. Nevertheless, the surface Cu is biologically available to animals (Suttle and Price, 1976) and it was estimated that if slurry residues constituted 2% of their daily dry matter intake for a period of 6 months, a hazard might exist (Dalgano and Mills, 1975).

Chronic Cu poisoning had frequently been encountered in housed lambs and sheep receiving large amounts of concentrates. Such diets may contain 40 mg Cu/kg or more when the diets have been stored or processed in contact with Cu salts used in pig rations and when Cu but not Mo is included in the mineral-vitamin mix (Underwood, 1981). In the latter circumstances complete sheep feed containing only 25 mg Cu/kg can cause Cu poisoning (Buck, 1970).

Symptoms and biochemical aspects of copper toxicity

The development of chronic Cu toxicity in sheep is generally regarded as occurring in two distinct phases (Ishmael et al., 1972).

First, there is a period of passive accumulation of Cu in the tissues. This period may vary from a few weeks to more than one year. During this phase the animal is apparently normal, showing no symptoms of toxicity. It is to this period that the term "chronic" really applies (Todd, 1976). The accumulation of Cu, predominantly in the liver and kidney, is accompanied by changes in these organs which are of greatest magnitude in the liver. Ishmael et al. (1972) recorded elevations of liver-specific serum enzyme levels, and a reduction in liver function in the prehemolytic phase of the disease. This was particularly marked in the 2 weeks prior to hemolysis. Histological and histochemical changes which have been recorded for this period include necrosis of liver parenchymal cells and the presence of swollen, periodic acid Schiff (PAS) positive, diastase resistant, Cu containing Kupffer cells which are rich in acid phosphatase (Howell, 1977).

The main clinical features of the second phase include jaundice, anorexia, excessive thirst and hemoglobinuria. Dramatic reductions in blood concentrations of hemoglobin and glutathione occur within 1 to 2 days, and there is a transient increase in blood methemoglobin concentration (Bremner, 1979). Death frequently results within a few days, although some animals may survive (Ishmael et al., 1972). The onset of these symptoms is associated with release of stored Cu from the liver and a massive increase in blood Cu concentrations.

At the time of hemolysis focal necrosis of liver tissue become extensive. Inflammatory cells, bile plugs and large PAS positive, diastase resistant Kupffer cells may be abundant. The activity of mitochondrial enzymes, such as adenosine triphosphatase and glutamic dehydrogenase, were found to be markedly reduced particularly in the central zones of the lobules, whereas the activity of the lysosomal enzyme, acid phosphatase, was increased in Kupffer cells and hepatic parenchymal cells (Howell, 1977).

The mechanisms underlying the changes in the liver are not yet clear. A number of studies indicate that excessive Cu accumulates in the nucleus and lysosomes. Howell (1977) found a marked increase in hepatic parenchymal cell lysosomes in the prehemolytic phase of chronic Cu poisoning in sheep, but Corbett et al. (1978) found no evidence of lysosomal accumulation of Cu in Cu-loaded sheep livers.

Considerable kidney damage also occurs at the time of the hemolytic crisis, with significant functional impairment and degeneration, necrosis, and loss of mitochondrial enzyme activity from the proximal convoluted tubules (Gopinath et al., 1974). Todd (1969) suggested that the appearance of the kidney, which is black and engorged with hemoglobin degradation products, is one of the most strongly characteristic features of Cu toxicity in sheep. It is thought that the renal failure and associated uremia play an important part in the death of sheep from Cu poisoning (Gopinath et al., 1974). Gooneratne et al. (1982) pointed out that kidney function was related to the degree of hemolysis and suggested that performing kidney function tests on the second day of the

hemolytic crisis made it possible to predict which animals would recover and which would die. That is, if the hypercupremia and hemoglobinemia were mild and present for less than 48 hours they would not cause marked impairment of kidney function.

Changes may also occur in other tissues, especially at the time of the hemolytic crisis. For example, there have been reports of spongy transformation and vacuolation of white matter of the brain (Morgan, 1973). Howell et al. (1974) suggested that these changes were because of the effect of altered metabolic processes on glial transport mechanisms. Muscle cell membranes may also be damaged, as increased levels of creatine phosphokinase have been detected in serum in the terminal stages of the disease (Thompson and Todd, 1974). This may have been brought about by a variety of factors including Vitamin E and selenium depletion of tissues (Gooneratne and Howell, 1980). Gooneratne and Howell (1982) have recorded in sheep, that accumulation of Cu in liver was associated with an increase in both Se concentration and glutathione peroxidase activity in that organ. They presumed that a depletion of Vitamin E occurs in Cu loaded sheep. Cu toxicity in sheep also causes an accumulation of spleen ferritin, which suggests that an alteration in ferritin may have occurred which affects iron deposition and release (Mertz et al., 1981).

The biochemistry of blood in chronic Cu poisoning has been extensively studied (McCosker, 1968b). Blood Cu levels remain normal until one to two days before clinical symptoms appear, and then increase to 5 to 20 times normal. At the time of hemolytic crisis Cu is released

from the liver into the blood stream and uptake of Cu by erythrocytes is essential for hemolysis to occur and that for this to happen the Cu must be in a direct reacting, TCA-soluble form (Gooneratne et al., 1981b).

Hemoglobin concentration falls rapidly over the next two to three days, and methemoglobin increases, reaching a peak in one to two days, and falls again, this pattern being similar to that for blood Cu. The other interesting feature is the dramatic fall in glutathione (Thompson and Todd, 1970). Metz and Sagone (1972) have proposed that the injury to the red blood cells stems primarily from accelerated oxidation of glutathione, with resulting oxidative injury to hemoglobin and the cell membrane.

Various increases in liver-related serum enzyme activities have been recorded 6 - 8 weeks before the hemolytic crisis. These enzymes include serum sorbitol dehydrogenase, acid phosphatase (Kumaratilake et al., 1982), lactic dehydrogenase, glutamic oxaloacetic transaminase, arginase and glutamic dehydrogenase (Hubbs and Oehme, 1982). The increased serum activities of these enzymes often subside to nearly normal levels 1 - 2 weeks before the hemolytic crisis, but very high levels of activity occur shortly before or during the crisis. It is important to note that these elevations were not correlated with increases of blood Cu level, which only occur shortly before and during the hemolytic crisis. Therefore they are of no diagnostic value before the animals fall sick (McCosker, 1968b). Determination of aspartate aminotransferase has been proposed as an aid in the diagnosis of chronic Cu toxicity in lambs (Buckley and Tait, 1981). During the hemolytic crisis, the activities of hydrolytic adenosine triphosphatase, nonspecific esterases and succinic dehydrogenase were reduced (Ishmael et al., 1972).

Factors influencing the development of copper toxicity

Copper poisoning is a complex problem because of the many factors which influence the metabolism of Cu. The onset of the hemolytic episode has taken place, for example, at liver Cu concentrations of 1000 (Tait et al., 1971) and 6000 mg Cu/kg DM (MacPherson and Hemingway, 1969). The incidence of Cu toxicity in sheep can not be related solely to the dietary Cu intake of the animals. Buck (1970) claimed that a dietary concentration of only 8 mg Cu/kg was sufficient to induce toxicity, but problems have arisen more commonly at intakes of 25 to 30 mg Cu/kg (Bremner et al., 1976).

Cu interacts metabolically with so many other elements, such as Mo, Zn, Fe and Cd, that it is impossible to give maximum safe or minimum tolerable dietary Cu levels based on Cu alone (Underwood, 1977). Froslie and Norheim (1983) concluded that an insufficient Mo intake caused the Cu toxicity in grazing sheep in Norway. Several investigations have been made on the protective effect of Mo against Cu poisoning in sheep, as this metal has a marked inhibitory effect on Cu availability, especially in the presence of sulfur (Dick, 1954). This is believed to be mediated through formation of thiomolybdates in the rumen (Dick et al., 1975).

The toxicity of Cu may also be influenced by the age, breed, and physiological state of the animal. For example, the hepatic retention of dietary Cu in young calves receiving liquid milk substitutes can be as great as 50% (Bremner and Dalgarno, 1973). It has been suggested that ewes are particularly susceptible to Cu poisoning in late pregnancy

arising from increased retention of Cu during pregnancy (Bremner, 1979). There may also be sex differences in susceptibility to Cu toxicity as Suttle (1977) found that mortality was higher for male lambs than for female lambs receiving the same Cu-supplemented diet, although liver Cu concentrations were similar in the two sexes. There is evidence that, sheep of the Texel breed were more affected than those of the Suffolk and Blackface (Woolliams et al., 1982). A further example of differences in susceptibility of breeds to Cu toxicity is provided by sheep of the Orkney breed from North Ronaldsay (Maclachlan and Johnston, 1982). Although these animals survive in their natural habitat on a diet consisting largely of seaweed and terrestrial herbage, it was found that they succumbed very readily to Cu poisoning when grazing on an upland farm regarded to be marginally Cu deficient. It was suggested that the capacity for highly efficient absorption and retention of Cu had developed in these sheep by natural selection over many generations as a consequence of the low availability of Cu in their natural feed of seaweed (Bremner, 1979).

Treatment or prevention of copper toxicity

Treatment of Cu toxicity after onset of the hemolytic crisis has proved difficult. However, preventive measures to prevent chronic Cu poisoning of the animals may be more successful. Such as replacing the diet with one of low Cu content. Daily oral treatment of affected lambs with 100 mg ammonium molybdate and 1 g anhydrous sodium sulphate significantly reduced the Cu content of tissues (Ross, 1970) and appeared to prevent deaths in lambs known to have taken toxic amounts of Cu. More

recently, Gooneratne et al. (1981a) have reported that intravenous injections of 100 mg ammonium tetrathiomolybdate twice weekly prevented the occurrence of hemolytic crisis in sheep repeatedly dosed with Cu-sulphate. This treatment also minimized tissue damage and prevented further hemolytic crisis when given to sheep already in hemolysis. These authors concluded that chronic Cu poisoning can be successfully prevented or treated by intravenous injection of appropriate doses of ammonium molybdate.

Increasing the dietary zinc concentration from 220 to 420 mg Zn/kg diet was found to be effective in reducing the incidence of Cu toxicity in growing lambs on a practical type of diet, with no serious side effects (Bremner et al., 1976). The concentration of Cu in sheep diets should never exceed 10 to 15 mg Cu/kg DM. Mo concentrations should also be monitored since Mo is an important interacting element (Hubbs and Oehme, 1982).

Copper deficiency in the ruminant animal

A wide variety of clinical abnormalities in sheep and cattle have been attributed to either a simple dietary deficiency of Cu, or conditioned deficiency resulting from the presence of a factor or factors which inhibit utilization and storage of Cu. These abnormalities are associated with low concentrations of Cu in blood and tissues and which respond to Cu therapy. Cu deficiency syndromes in cattle occur most frequently in young calves; symptoms include scouring, unthriftiness, loss of coat color, and abnormalities in bones of the limbs. Further, there is a condition, confined to Australia, termed "falling disease"

which is characterized by atrophy and fibrosis of the myocardium which progresses to such an extent that the animal suddenly drops dead. In sheep the processes of pigmentation and keratinization of wool are the first to be affected by a lowered Cu status, followed by enzootic ataxia usually of delayed form after more prolonged deficiency.

Occurrence

Cu deficiency is essentially a disease of grazing livestock subsisting on natural forages. The classical situation is that of beef cattle on low-cost production systems reliant upon home-grown foodstuffs, mainly grass or silage. The feeding of dry feedstuffs, especially of concentrates normally rich in Cu, prevents the possibility of any problem in most ruminant animals. Hypocupremia in the grazing animal can arise (a) from a low dietary intake of Cu (Lewis, 1975) or (b) when the herbage has an apparently adequate Cu content, but the presence of factors interfering with the absorption, utilization or storage of Cu (Drysdale, 1979; Merry et al., 1983) or (c) as a combination of these effects particularly under winter grazing conditions when the total intake of nutrients, including Cu, may be low (Suttle et al., 1975).

The manifestation of Cu deficiency also depends upon the age of animal (Clegg et al., 1983), breed (Wiener and Field, 1969; Mills, 1982) sex and species of the animal (Underwood, 1977; Roberts, 1976) and with the severity and duration of the deficiency (Lewis, 1975; Underwood, 1977).

Retarded growth and weight loss

The effect of Cu deficiency most frequently responsible for economic loss is the decline in growth rate occurring in calves and yearlings when a deficiency becomes established (Camargo et al., 1982; Hennig et al., 1974). Poole et al. (1974) have indicated that in clinical Cu deficiency, growth patterns of beef cattle five months to one year of age, were affected and resulted in lower yield of fat and muscle. There was no effect on performance with Cu depletion occurring after one year of age. A growth response to Cu during induced Cu deficiency has been demonstrated in sheep (Hogan et al., 1971) and in experimental lambs fed a diet containing 0.3 mg Cu/kg DM (Howell, 1968).

The small intestine of animals undergoing Cu-depletion has been shown to suffer marked cellular damage that is probably of sufficient severity to affect absorptive function and such events may well underlie the marked decline in efficiency of food utilization (Fell et al., 1975). Loss of weight, poor growth and emaciation are probably related to the earliest and most severe biochemical lesion in Cu deficiency, that is, the rapidly progressive loss of cytochrome oxidase activity (Gallagher, 1979). The high metabolic demands of young growing animals are progressively not met because of diminishing cytochrome oxidase activity with Cu depletion. This results in the clinical signs of retarded growth and cessation of growth far below the normal mature weight. Continued depletion of Cu and loss of cytochrome oxidase activity restrict oxidative metabolism below the body maintenance level, leading to loss of weight (Gallagher, 1979).

Scouring or diarrhea of cattle

Scouring or diarrhea of cattle associated with Cu deficiency or excess Mo has been reported in several parts of the world (Havre, 1970; Camargo et al., 1982). The appearance of marked diarrhea in Cu deficient cattle provides further evidence of a key role for Cu in maintenance of the normal function of the gastrointestinal tract. One of the clinical signs of Cu deficiency in animals grazing on pastures high in Mo is profuse diarrhea (Bull, 1980; McMurray, 1980). The diarrhea produced by high concentrations of Mo could be because of either a much more severe Cu deficiency or to the presence of Cu, S and Mo complexes that exert direct toxic effects on the gut mucosa, as seen in the rat (Fell et al., 1979; McMurray, 1980). Fell et al. (1975) reported diarrhea with mucosal atrophy in the small intestine, partial villus atrophy, elongation of cryts and goblet cell hyperplasia in Cu deficient steers. These changes together with diminished cytochrome oxidase activity in the small intestine may have produced a malabsorption syndrome resulting in decreased food conversion and diarrhea.

Considerable evidence indicates that noradrenaline markedly affects intestinal motility and Davies et al. (1982) suggested that the cause of the diarrhea could be changes in tone of the intestinal musculature. Accordingly, in their experiments on Cu deficiency in cattle, they observed 41% less noradrenaline in duodenal muscle and 59% less in colonic muscle, compared to control animals. The Cu deficiency caused no changes in noradrenaline concentrations of the mucosa from either intestinal site. However, the significance of their observation in relation to the

diarrhea in Cu-deficient cattle has yet to be established.

Paynter and Allen (1982) found that Cu-superoxidase dismutase activity accounts for a large proportion of the cellular Cu at several sites along the intestinal tract and that this activity is responsive to dietary Cu. These authors suggest that changes in this enzyme should also be considered in relation to the mechanisms involved in enterocyte mitochondrial abnormalities and villus atrophy changes associated with Cu-responsive diarrhea in cattle.

Impaired keratinization and depigmentation of wool and hair

Changes in the growth and physical appearance of hair or wool have been noted in Cu-deficient cattle and sheep. Abnormalities of the wool are the first observed sign and may be the only sign in marginal Cu deficiency. Fine wool becomes limp and glossy and loses its crimp, developing a straight, steely appearance (Blood et al., 1979; Bull, 1980). The tensile strength and other properties of wool are adversely affected by the deficiency state (Bull, 1980). The capacity to impart crimp begins to be influenced when blood Cu concentration falls below 40 µg/100 ml and fails completely below 20 µg/100 ml if these levels are maintained over many months. Supplements of 7.5 - 10 mg Cu/day are sufficient to prevent the condition (Lewis, 1975). The characteristic physical properties of wool, including crimp, are dependent on the presence of disulphide groups that provide the crosslinkage or bonding of keratin and on the alignment or orientation of the long-chain fibrillae in the fibre. Both of these are adversely affected in Cu deficiency but the precise biochemical involvement of Cu is unknown (Underwood, 1981).

A major effect of Cu deficiency in sheep is depigmentation of black-fleeced animals, and a reduction in the amount and quality of the wool (Smith and Gawthorne, 1975; Hogan et al., 1971; Rish, 1970). Lack of pigmentation in the wool of sheep is a more sensitive index of Cu deficiency than anemia (Underwood, 1977; Coelho da Silva, 1978). The pigmentation process in the sheep is so sensitive to changes in Cu intake that alternating bands of unpigmented and pigmented wool fibres can be produced, as Cu is withheld from and added to the diet (Underwood, 1981).

Loss of hair pigment is seldom a clinical feature of Cu deficiency in cattle (Fell et al., 1975). Bleaching of the fleece has been noted, particularly in lambs, maintained on semi-purified, low Cu diets, but greying of the hair and achromotrichia (i.e. loss of hair color) are usually associated with the interaction of Mo and sulfate (Lewis, 1975).

The exact mechanism involving the function of Cu in the process of pigmentation is unknown. It is possible that a Cu-containing enzyme, polyphenyl oxidase, catalyzes the synthesis of melanin from L-tyrosine at the wool or hair follicle, and when deficient the pigment for black is not incorporated into the follicle. This results in a whitening of hair or wool (Bull, 1980).

Neonatal ataxia

A nervous disorder of newborn and young lambs, and less frequently of cattle, which is characterized by incoordination of movement and associated with low levels of Cu in the tissues is well recognized in many parts of the world. Two forms are recognized: (1) congenital in

which the lambs are affected at birth with macroscopic cerebrospinal lesions; and (2) a delayed form in which symptoms may appear from about 1 to 12 weeks after birth (McMurray, 1980; Lewis, 1975). Neonatal or enzootic ataxia in Australia or swayback in Great Britain has always been associated with low Cu concentration in blood, liver and brain (Hennig et al., 1974; Chamberlain and Clarke, 1981; Brightling, 1983) and can be prevented by Cu supplementation of the dam during pregnancy (Butler and Barlow, 1963; Chamberlain and Clarke, 1981). The delayed condition has been reproduced experimentally in primary (Suttle et al., 1970) and in secondary Cu deficiencies induced by Mo and sulfur (Alloway, 1973).

Adequate maternal intake of Cu is essential for development of the central nervous system of the embryonic lamb. Consequences of Cu deficiency during intrauterine life may include gross brain lesions, with affected lambs born dead or dying shortly after birth (Hidiroglou and Knipfel, 1981).

In affected animals the lesions causing this condition occur in the brain and spinal cord. The lesions which are consistently found are in the myelin of the spinal cord and have been described as demyelination, myelin aplasia, or dysmyelinogenesis (Howell, 1970). The last two terms are probably more accurate, since the lesions develop before or shortly after birth when proliferation of the central nervous tissue is very rapid (Todd, 1976).

Howell and Pass (1982) suggested that the various lesions seen in swayback animals are all because of Cu deficiency and that the variation depends on the existence of a critical Cu deficiency at the time when

a particular structure is at its most vulnerable stage of development. If a) the critical period of Cu deficiency occurred in early or mid pregnancy the cerebral hemispheres would be more vulnerable, b) the critical period of Cu deficiency occurred in late pregnancy myelination might be affected in the brain and spinal cord and cellular multiplication and division may be affected, particularly in the cerebellum, producing a lesion in myelinated structures in cerebrum and cord and changes in granule and purkinje cells in the cerebellum, c) the critical period of Cu deficiency occurred after birth the spinal cord would be most severely affected. This is comparable to the situation in the delayed case.

The major biochemical lesion in the brain of an affected animal appears to be a reduction in the Cu-containing respiratory enzyme, cytochrome oxidase related to low levels of brain Cu (Howell, 1970; Hurley and Keen, 1979). Gallagher and Reeve (1971) reported that there is a causal relationship between low cytochrome oxidase activity and phospholipid production in the liver mitochondria of Cu deficient rats. Apparently, the low activity of cytochrome oxidase is not sufficient for the production of the ATP needed for phospholipid synthesis. If a similar effect occurs also in brain, the myelination seen there could result from a lack of the phospholipids needed for myelin synthesis (Gallagher, 1979).

Anemia

Anemia is one of the manifestations of Cu deficiency in ruminant

animals where the deficiency is severe or prolonged. If levels of Cu in the blood fall from the normal 0.80 - 1.20 µg Cu/ml to as low as 0.10 - 0.20 µg Cu/ml, normal hematopoiesis cannot be sustained (Underwood, 1981). The induction of anemia as a result of simple Cu deficiency has been demonstrated by Howell (1968) using semi-purified low Cu diets, but the incidence and severity depend on the Cu intake, duration of deficiency and physiological status of the animals. Suttle et al. (1970) using a diet containing 1.20 µg Cu/g found no anemia in ewes, during pregnancy or after parturition. Certain animals seem more prone to anemia than others, and the occurrence and severity is not uniformly correlated with blood and liver Cu concentrations, or presence of ataxia in the lambs. Parenteral administration of small amounts of Cu (5 - 10 mg) rapidly restores hemoglobin levels to normal although producing only a small transient rise in blood Cu concentration (Lewis, 1975).

The morphological characteristics of the anemia occurring from Cu deficiency varies with the species. In lambs and calves it is hypochromic and microcytic (Underwood, 1977; Smart et al., 1981), and in cattle and ewes it is hypochromic and macrocytic (Coelho da Silva, 1978). The type of anemia associated with Cu deficiency is identical to that caused by iron deficiency. Moreover, animals fail to respond to a diet deficient in Cu if given adequate amounts of iron orally or parenterally. Their mucosal epithelial cells, hepatic parenchymal cells, and reticuloendothelial cells are able to take up iron normally, but they are unable to release iron to the plasma at the normal rate (NRC, 1977).

Copper is an essential component of adult red cells and a certain minimum must be necessary both for their production and for the main-

tenance of their integrity in the circulation. Cu does not appear to be involved in the heme biosynthetic pathway but is essential for the mobilization of iron from the tissue and its utilization in hemoglobin synthesis. These functions are accomplished by ceruloplasmin, the Cu-containing metalloenzyme, ferroxidase of the plasma. This enzyme is necessary for the formation of Fe^{3+} transferrin, the transport vehicle for Cu (Underwood, 1981). The iron in this compound is contributed directly to reticulocytes in the bone marrow. A high rate of metabolism in bone marrow is essential for normal erythropoiesis.

The erythrocytes in Cu deficient animals have a shorter survival time than normal. This could be related to the toxic effect of the superoxide anion, O_2^-, a highly reactive free radical produced by the oxidation of some substrates by molecular oxygen. Decomposition, and thus detoxification, of the superoxide anion is catalyzed by the cuprozinc enzyme, superoxide dismutase, which is present in red blood cells (Gallagher, 1979). Suttle and McMurray (1983) have shown that erythrocyte superoxide dismutase (erythrocuprein) activity was reduced in Cu deficient ruminant animals.

Bone disorders

Although bone abnormalities have been reported for various species, they are not a common characteristic of Cu deficiency in ruminants. A low incidence of spontaneous bone fractures has been observed in sheep and cattle grazing Cu deficient pastures (Suttle et al., 1972; Smart et al., 1981), but the most prominent sign is a very marked stiffness of the legs (Smart et al., 1980). Cu deficient animals may show signs

of rickets with beading of the ribs and enlargement of the ends of the long bones.

Histologically, the affected bones show a widening of the growth plate and the overall appearance of the lesion is that of osteoporosis (Smart et al., 1980; Hidiroglou, 1980). The lesion is most severe in the central metaphyseal region and is consistent with a matrix osteoporosis arising from a reduction in or cessation of osteoblastic activity (Suttle et al., 1972; Underwood, 1977). Suttle et al., (1972) suggested that osteoporosis can be a consequence of simple Cu deficiency in lambs born to Cu depleted ewes and that osteoblastic activity is one of the first functions to be impaired. The osteoblast is apparently more sensitive to Cu deficiency in fetal and neonatal lambs than in older lambs since weaned lambs which were reared on the same diet and became hypocupremic at 5 months of age had no evidence of osteoporosis or depressed osteoblastic activity after a further 8 months depletion (Suttle et al., 1972).

The primary biochemical lesions in the bones of Cu deficient animals is probably a reduction in the activity of the Cu-enzyme amine or lysyl oxidase. This leads to diminished stability and strength of bone collagen as a result of impaired crosslinking of its polypeptide chains (Underwood, 1981). It is important to note that, although Cu supplementation can arrest this syndrome (Smart et al., 1980), the damage that occurred before supplementation is irreversible (Hurley and Keen, 1979).

Cardiovascular disorders

Cardiac lesions have been noticed in Cu-deficient cattle, with atrophy of the myocardium and the occurrence of fibrosis (Davis et al., 1974). The pathological process is a progressive one, extending over a period of years and proceeding to the replacement of large areas of atrophied myocardium with dense collagenous tissue (Coelho da Silva, 1978). Sudden death is believed to be due to acute heart failure, usually after mild exercise or excitement. This disease does not occur in sheep grazing the same Cu deficient pasture (Underwood, 1981).

The primary biochemical lesion is thought to be a decrease in lysyl oxidase activity. This enzyme, for which Cu is a cofactor, catalyzes the oxidation of certain peptidyl lysine and hydroxy-lysine residues to peptide aldehydes, which initiate a crosslinking mechanism required for connective tissue stability (Rucker and Tinker, 1977). Copper deficiency leads to decreased lysyl oxidase activity, with a concommitant decrease in elastin crosslinks (Hurley and Keen, 1979), but Davies et al. (1982) found that lysyl oxidase activity of the aorta was unaffected in Cu-deficient steers. Gallagher (1979) stated that heart muscle is a very active tissue which requires a high level of cytochrome oxidase activity to maintain its oxidative metabolism. Cytochrome oxidase in the myocardium is severely depleted in Cu deficiency. Consequently, the extra oxidative demand made of the heart by forced exercise, when most deaths of Cu-deficient cattle occur, may exceed the available oxidative capacity and result in repsiratory failure, leading to focal or generalized myocardial necrosis. More recently, Paynter and Allen (1982) did not sup-

port this statement as they found that Cu-deficiency reduced cytochrome oxidase in several tissues but not in heart, even though Cu concentrations were reduced in this tissue. They indicated that changes in factors other than heart cytochrome oxidase activity may be involved in this tissue. More research is needed to elucidate the causes of the heart lesions.

Infertility

Low fertility in cattle grazing Cu-deficient pasture, associated with delayed or depressed oestrus, occurs in several widely separated geographical areas (Underwood, 1981). Infertility in ewes, associated in some cases with aborted small dead fetuses, has been reported in experiments using a diet of very low Cu content (Howell, 1968; Howell and Hall, 1970). However, other experiments, using low Cu diets (1 - 3 mg Cu/kg DM) have not suggested any relationship between Cu deficiency and conception rate in ewes (Suttle et al., 1970).

The nature of possible reproductive problems is not clear, but the developing fetus suffers from anemia and exhibits hemorrhages, resulting in mortality due to a lack of adequate synthesis of elastin (and collagen) for normal embryonic development (Bull, 1980).

Treatment and prevention of Cu deficiency

The effectiveness of treatment of a Cu deficiency depends on whether the changes which have already occurred in animal tissues are reversible. In Cu deficiency there is a good evidence that both reversible and irreversible changes occur. Diarrhea is reversible owing to the continual

turnover of cells in the gut mucosa; hair colour is reversible because of the continuous production of hair and presumably because the enzyme systems responsible change when Cu status changes. In contrast, swayback in lambs is irreversible, and Cu appears to be necessary at key times in the formation of the myelin sheath (McMurray, 1980).

The prevention of a deficiency requires more than the provision of an abundant uptake of the Cu by oral or parenteral supplementation. With elements such as Cu, the level of supplementation must be carefully assessed because there is a relatively narrow margin between intakes that cause deficiency and toxicity especially in sheep.

Much trace mineral supplementation has been on an individual animal basis, though application of the minerals in fertilizer is widespread. Effective methods of supplementation of particular trace minerals depend to a large extent on whether or not they are stored in the body. Cu and Zn are stored in a number of tissues, but principally in the liver, therefore supplementation need only be done at intervals, as any deficit between demand and normal dietary supply can be met from body reserves. On the other hand, Mo is not stored to any appreciable extent in the body so supplementation should, at best, be daily. This can be achieved by fertilization of pastures; by salt licks; by pulse dosing (orally or by injection); or by slow release devices located within the body of the animals.

The application of Cu-containing fertilizers can be an effective means of raising the Cu content of the herbage to levels adequate for grazing stock and also will often increase herbage yields. The amounts required vary with the soil type and climatic conditions. A single

dressing of 5 - 7 kg/ha of copper sulfate, or its Cu equivalent in the form of cheaper Cu ores, is usually sufficient for 3 - 4 years (Underwood, 1981). Hannam et al. (1982) suggested that a Cu dressing of 2 kg/ha to sandy soils provides adequate Cu for pasture and sheep production for at least 23 years and that repeated dressing is unwarranted.

Foliar application of 5.6 - 11.2 kg $CuSO_4$/ha in solution to pasture or haylands increased the Cu concentration in forage to 100 and 208 ppm respectively (MacPherson et al., 1975). Forage with such a high Cu content would be toxic to ruminant animals, but rainfall within 24h of the time of application, washed the $CuSO_4$ off the forage and reduced the Cu concentration to 24 and 41 ppm respectively. MacPherson et al. (1975) suggested that the application of 1.1 kg $CuSO_4$/ha in dry conditions is sufficient to raise Cu concentrations in hay to levels which should preclude the occurrence of Cu deficiency when fed to cattle in winter.

Under range conditions where fertilizer treatments are uneconomical, or on calcareous soils where Cu absorption by plants is poor (Underwood, 1977), deficiency can be prevented by the provision of Cu-containing salt licks, by dosing or drenching the animals at intervals with Cu compounds or by injection of organic complexes of Cu. Mineral mixtures, or salt licks, containing 0.25 - 0.5% of copper sulfate for sheep (Blood et al., 1979) and 2.5 - 5.0% (Smart et al., 1980) for cattle will supply sufficient Cu provided adequate intake of the mixture is assured. However, it is impossible to control intake by individual animals.

Another route of Cu supplementation for animals or pasture could be by adding Cu salts to drinking water. The addition of 2 - 3 mg Cu/litre

of water (MacPherson, 1982; Farmer et al., 1982) maintained normal blood Cu levels in normal grazing seasons, but precipitation and seasonal differences in the quantity of water consumed are disadvantages.

In intensive production systems, Cu can be added to supplemental feed. Addition of $CuSO_4$ to concentrate diets to provide 10 - 15 ppm Cu increased the plasma Cu of dairy calves and bulls to normal level (Maro and Kategile, 1980; Steacy et al., 1983).

Periodic parenteral injections of Cu compounds, which release Cu gradually, have provided good results and have the advantage of avoiding fixation of Cu by Mo in the alimentary tract (Suttle and Field, 1974). The parenteral treatment of Cu deficiency in ruminants is widely practiced but it has three disadvantages: reaction at the site of injection (Boila et al., 1982; Ward and Nagy, 1976); risk of acute general toxicity (Ishmael et al., 1970; Wasfi and Adam, 1976); and the need for repeated injections (MacPherson et al., 1979).

The injection of 45 mg Cu (as Cu glycine); 40 mg Cu (as Cu methionate) or 50 mg Cu (as CuCaEDTA) given to ewes in mid-pregnancy increased mean blood Cu concentrations. Such parenteral injection of the ewe was also effective in raising lamb blood and liver Cu concentrations (Hemingway et al., 1970). Subcutaneous injection of 90 mg Cu, as Cu methionate, in ewes increased milk Cu concentration, and resulted in transitory increases in lamb plasma Cu concentration and rate of liveweight gain (Whitelaw et al., 1981; Whitelaw and Evans, 1979). The injection of 80 mg Cu by intramuscular injection at 6-week intervals was adequate to maintain normal blood Cu status and increase liveweight gain in steers (MacPherson and Dixon, 1980).

Mahmoud and Ford (1981) conducted a trial to compare three different organic compounds of Cu given by sheep. A) 6 mg Cu/kg body weight (B.W.) as Cu-methionate; B) 3 to 4 mg Cu/kg B.W. Cu as CuCaEDTA and C) 2 mg Cu/kg B.W. as dielthylamine copper oxyquinoline sulfate (CuDOS). The results indicated that compound (A) produced no deleterious effect, compound (B) caused death of the sheep, while compound (C) produced liver and kidney lesions. The authors suggested that rapid absorption and transfer of Cu to the liver and kidneys may have been responsible for the toxic effects of compounds (B) and (C).

Suttle (1981b) in a similar experiment compared four parenterally administered Cu complexes to alleviate hypocupremia in sheep and cattle. He found that, at similar dose rates, Cu as CuDOS more consistently alleviated hypocupremia in ewes than CuCaEDTA or Cu-methionate given in a cream (c) or aqueous base (a). In similar comparisons of preparations for cattle, CuCaEDTA was 19% more effective than CuDOS and 36 to 48% more effective than Cu methionate (a). Methionate preparations were characterized by marked reactions at, and slow translocation from the site of injection, whereas CuDOS was rapidly translocated and gave little or no tissue reaction in sheep and cattle.

Following comparison of different routes of injecting Cu complexes, Lewis et al. (1982) suggested that the subcutaneous injection is preferrable to intramuscular injection for young lambs.

In a summary, the treatment of Cu deficiency by parenteral injection, using chelates which are currently available, methionates and glycinates were judged to be slowly translocated from the site of injection and give the largest local reactions (Boila and Devlin, 1982).

Deep intramuscular administration of these compounds merely conceals the problem and may cause condemnation of valuable parts of the carcass. The CuDOS complex causes no local reaction, but can be acutely toxic and gives relatively shortlived protection at the small doses which are recommended (Suttle, 1983). CuCaEDTA may have the least disadvantages of the commonly used chelates.

Oral Cu therapy has in the past been an inferior alternative to parenteral treatment. If given to sheep and cattle on pasture as cupric sulfate less than 2.4% of an oral copper dose is retained in the liver (ARC, 1980). Little attention has been given to the possibility that other preparations of Cu might be more effective. A new method of long-acting supplementation has now been developed which eliminates the carcass damage often associated with parenteral injections. This involves the administration of oxidized copper wire (Judson et al., 1982) or copper oxide granules or needles (Suttle, 1981a), of high specific gravity and low mass, in gelatin capsules into the oesophagus with a tube or balling gun.

The Cu oxide needles or particles are retained in the abomasum of sheep (Dewey, 1977) and in the reticulo-rumen and abomasum of cattle (Suttle and Valente, 1981) where Cu is released over a period of several weeks. In the acid medium of the abomasum trace amounts of Cu are solubilized in ionic form, and absorbed from the gut to meet current Cu requirements, or to be stored in the liver. The remainder of the needles are eventually excreted in the feces. The practical success of these forms of supplementation depends on the length of time particles are retained in the abomasum and reticulo-rumen (Murphy et al., 1982;

Judson et al., 1982).

The oral administration of a dose of cupric oxide needles providing 0.5 g Cu, to hypocupremic ewes maintained on a Cu-deficient diet, alleviated hypocupremia for 111 days (Suttle, 1981a). In a further experiment with hypocupremic steers and heifers, the administration of a dose of cupric oxide needles, providing 40 g Cu, alleviated hypocupremia for not less than 41 days, at which time a substantial reserve of Cu (428 mg) remained in the liver (Suttle, 1981a).

Technology has now reached the stage where, in the near future, it will be possible to administer many trace minerals by controlled release intraruminal devices. A general purpose capsule (Fig. 1) can be used for single or multi element supplementation. The wings of the

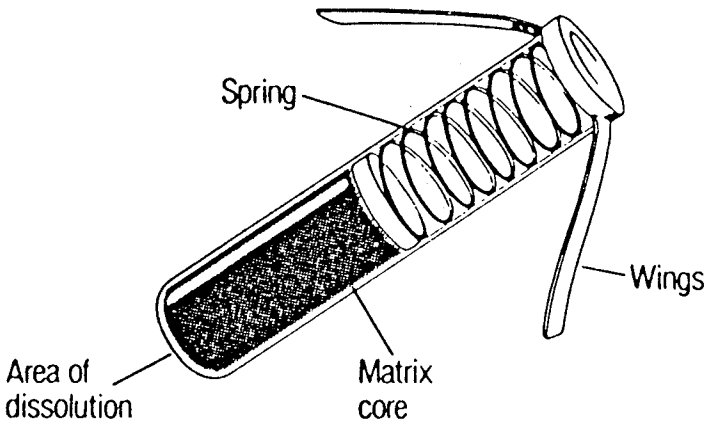


Figure 1. Illustration of general purpose capsule showing wings in expanded state (Adapted from Siebert and Hunter, 1982).

capsule open in the rumen to prevent regurgitation. The concept of the general purpose capsule is simple. The capsule consists of a hollow plastic cylinder containing a core of matrix. The supplemental mineral

is homogenously dispersed through the matrix. As the carrier matrix dissolves at the open end, the core is moved towards the orifice by the spring to maintain a constant area for dissolution. Thus an approximately constant release rate is achieved (Siebert and Hunter, 1982).

Copper requirement

The copper requirements of ruminant species cannot be precisely defined because various interfering factors may be present in the diet. The level of dietary Cu required for health is somewhat species-dependent and usually positively correlated with dietary levels of Mo and S (Ammerman et al., 1980). If Mo content of the diet is less than 1 mg/kg DM, the optimal level of Cu is between 6 and 8 mg/kg. Cu at 8 mg/kg DM is inadequate when Mo is between 1 and 3 ppm of the diet and can result in deficiency symptoms. Pastures containing 20 or more mg Mo/kg present an ever greater need for supplemental Cu in the diet (Bull, 1980). Barry et al. (1983) found that, pastures of kale forages, which contain low Cu concentrations (4 mg/kg DM) and high S concentrations (8 g/kg DM), caused lower serum and liver Cu concentrations in growing sheep and cattle than ryegrass-clover forage. These authors suggested that Cu requirement must be greater for growing sheep and cattle grazing kale than ryegrass-clover pasture.

Several investigators have used semipurified diets to study Cu requirements of ruminants. Suttle and Field (1968) fed semi-purified diets containing 1 or 11 ppm of Cu and/or 50 ppm of Mo and 1% sulfate. The 1 ppm of Cu was not sufficient to prevent a marked decline in plasma Cu and a lower than normal level of blood Cu in the lambs. The 11 ppm

Cu was apprently adequate to maintain normal blood levels, but not in the presence of the supplemental Mo and sulfate.

Dietary Cu requirement is based on the quantities contained in the tissues or secretions produced in various physiological states, and the endogenous losses from the body. The sum of these factors yields the physiological Cu requirement at the tissue level, known as the net requirement (ARC, 1980). Tables 1 and 2 show that the requirements of both species vary with growth rate and stage of lactation. It is interesting to note that the predicted requirements for cattle are approximately double those for sheep but relatively constant for different classes of animals (ARC, 1980). Smith (1982) suggested that the endogenous loss of Cu is more related to body size than to metabolic rate of the animal.

Therefore in view of the foregoing, additional information is required to better define the interactions among Cu, Mo and S which arise in the intestinal tract and in the blood, to determine antagonistic reactions function by reducing Cu absorption, or by restricting utilization of absorbed Cu. The present study was carried out to determine: a) the metabolism of Cu in the presence of Mo and S. b) Effect of Mo and S on Cu absorption and excretion. c) The ratio of Cu to Mo in the presence of adequate S necessary to maintain Cu balance in sheep.

Table 1. Estimated requirements of sheep for Cu* (ARC, 1980)

Class of animal	Live weight (kg)	Rate of gain, stage of pregnancy or milk yied	Relative requirement (mg Cu/kg diet DM)
Growing lamb (castrate)	5	0.150 kg/day gain	1.0
	10	0.150	1.0
	20	0.150	1.7-1.9
	40	0.075	2.7-4.5
		0.150	2.6-4.6
		0.300	5.1
Adult	50	0	4.6-7.4
Pregnant ewe (twin foetuses)	75	last 7 weeks	6.2-7.5
Lactating ewe	75	1 kg milk	4.6-5.8
		2	4.4-5.6
		3	5.6-8.6

*Calculated from the following components: endogenous loss, 4 µg/kg live weight (LW); growth, 1.15 mg Cu/kg LW gain; milk, 0.32 mg Cu/kg (early lactation); pregnancy, 0.32 mg Cu/day; wool, 5 mg Cu/kg DM.

Table 2. Estimated requirements of cattle for Cu* (ARC, 1980)

Class of animal	Live weight (kg)	Rate of gain, stage of pregnancy, or milk yield	Relative requirement (mg Cu/kg diet DM)
Pre-ruminant calf	40	0.5 kg/day gain	1.2
Growing cattle	100	0.5	8.1
		1.0	10-16
	200	0.5	8-14
		1.0	9-15
	300	0.5	8-15
		1.0	9-15
Adult	500	0	12-19
Pregnant cow	500	7 months	13-17
		9	13-20
Lactating cow	500	10 kg milk	10-14
		20	8-11
		30	8-11

*Calculated from the following component: endogenous loss, 7 µg Cu/kg live weight (LW)/day; gain, 1 mg Cu/kg gain; milk, 0.1 mg Cu/kg milk; pregnancy, increment of o.61 to 2.07 mg Cu/day from day 140 to 281.

MATERIALS AND METHODS

Animals and management

Twenty-eight Suffolk sired ram lambs at 125 (±23 SD) days of age and weighing 34.7 (±6.60 SD) kg were assigned on the basis of body weight to one of four treatment groups such that there were seven ram lambs per treatment in a split-plot design. All lambs used in the experiment were born from February to May, 1982, weaned at six weeks of age and raised in confinement with their dams. Lambs had access to pelleted creep feed and alfalfa-brome hay for three months after birth. The lambs and ewes were then moved to an alfalfa-brome grass pasture for a period of four weeks.

On August 3, 1982, at the beginning of the experimental period, the lambs were moved to a barn, allotted to four pens each equipped with one two-animal grain feeder and had access to tap water. Housing consisted of sheltered pens on concrete flooring with wood chip bedding that was changed every second day. Ten weeks later, at the beginning of cold weather (on October 12, 1982) the animals were moved to an enclosed area equipped with a heating system and each pen had one nipple-type tap water supply.

One lamb of average body weight (43.7±1.03 kg) from each treatment diet, was selected for a detailed Cu and Mo balance trial. The selected lambs were placed in metabolism crates for periods of five days for urine and feces collection (Fig. 2). After the collection period was over, the lambs were returned to their respective pens for 23 days until the next collection period.

Diets

The treatment diets were fed to lambs ad libitum in a pelleted from and water was available at all times. The premix of the pelleted diets was supplemented with either anhydrous sodium sulfate (Na_2SO_4) and ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$) alone or with copper sulfate ($CuSO_4\cdot 5H_2O$). The four treatments were: no added Cu, Mo or S (Control); 0 Cu + 10 mg Mo/kg + 2 g S/kg (0 Cu); 10 mg Cu/kg + 10 mg Mo/kg + 2 g S/kg (10 Cu); and 20 mg Cu/kg + 10 mg Mo/kg + 2 g S/kg (20 Cu) as shown in Table 3. Table 4 indicates the composition of diets fed to the lambs for 16 weeks. All feed was mixed in 250 kg batches in seven different mixes and stored in feed bags. All feed was weighed before being added to the feeders. Grab samples from each feed bag were collected to provide a composite sample for each batch for nutrient analysis. These composite samples were analyzed for Cu, Mo, S concentrations and Cu:Mo ratios were calculated (Table 5).

Sampling procedure

During the experimental period of 16 weeks, body weight, blood samples and liver biopsies were obtained on the first day and at the end of each subsequent four week period (Fig. 2). Body weights were recorded the day before blood samples and liver biopsies were obtained. Blood samples for serum were taken from the jugular vein and kept at $4^{o}C$ in a cooler for 12 hrs. They were then centrifuged for 30 minutes at 8000 g and two ml of serum was transferred by an air displacement pipette into previously acid washed vials for immediate wet ash

Table 3 Experimental design for ram lambs fed pelleted diets for 16 week

Treatment	Cu (mg/kg)	Mo (mg/kg)	S (g/kg)
Control	--	--	--
0 Cu	--	10	2
10 Cu	10	10	2
20 Cu	20	10	2

Added Cu as $CuSO_4 \cdot 5H_2O$ Added S as Na_2SO_4

Added Mo as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$

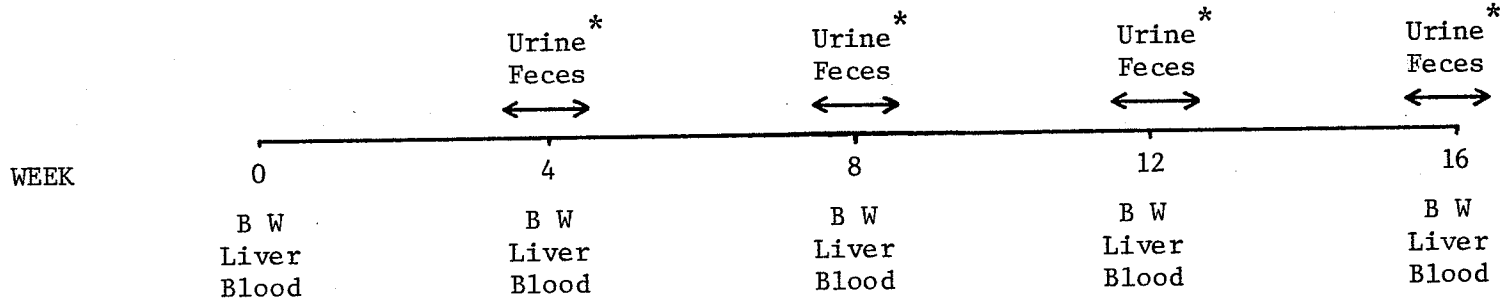


Fig. 2. Sampling schedule for body weight (B W), blood, liver biopsy, urine and feces collection for ram lambs fed pelleted diets for 16 weeks.

*five day collection period

Table 4. The composition of the pelleted diets fed ad libitum to ram lambs for 16 weeks

Ingredient	Treatments			
	Control	0 Cu	10 Cu	20 Cu
Chopped alfalfa-brome hay (kg)	78.0	78.0	78.0	78.0
Crushed barley (kg)	158.0	158.0	158.0	158.0
Molasses (kg)	2.9	2.9	2.9	2.9
Cobalt-iodized salt (kg)	1.1	1.1	1.1	1.1
Premix:				
Copper sulfate[1](g)	0.0	0.0	9.375	18.75
Anhydrous sodium sulfate[1](kg)	0.0	2.2	2.2	2.2
Ammonium molybdate[1](g)	0.0	4.6	4.6	4.6
Urea[2](kg)	1.25	1.25	1.25	1.25
Vitamin A (IU)	5×10^5	5×10^5	5×10^5	5×10^5
Vitamin D (IU)	4×10^4	4×10^4	4×10^4	4×10^4
Vitamin E (IU)	550.0	550.0	550.0	550.0
Wheat middlings (kg)	8.75	6.55	6.54	6.52
Total (kg)	250.0	250.0	250.0	250.0

[1]Copper sulfate @ 25% Cu, Anhydrous sodium sulfate @ 22.57% S and Ammonium molybdate @ 54.34% Mo.

[2]Urea was not added to diets the last 7 weeks of the experiment and was replaced by wheat middlings.

Table 5 Analyzed Cu (mg/kg), Mo (mg/kg), S (g/kg) content (DM) and Cu:Mo ratio of pelleted diets for each batch of feed used during the experiment

Diet	Mineral	1	2[ab]	3[b]	4[a]	5[b]	6[a]	7[a]	Mean ± SE
Control	Cu	12.3	10.8	18.2	8.7	15.5	6.9	8.3	11.5 ± 1.5
	Mo	1.0	4.3	5.4	2.1	3.1	1.6	2.1	2.8 ± 0.6
	Cu:Mo	11.8	2.5	3.3	4.2	5.0	4.3	4.0	5.0 ± 1.1
	S	2.3	1.8	1.8	1.8	1.8	1.8	1.6	1.8 ± 0.07
0 Cu	Cu	8.2	7.1	9.2	6.3	8.1	7.4	6.8	7.6 ± 0.4
	Mo	9.1	10.2	11.8	10.9	11.5	12.1	12.9	11.2 ± 0.6
	Cu:Mo	0.9	0.7	0.8	0.6	0.7	0.6	0.5	0.6 ± 0.05
	S	3.3	3.5	3.2	3.3	2.9	3.4	3.2	3.3 ± 0.07
10 Cu	Cu	16.7	16.1	17.8	16.4	16.4	17.8	14.9	16.5 ± 0.4
	Mo	8.7	7.8	11.0	16.2	11.3	13.3	15.3	11.9 ± 1.2
	Cu:Mo	1.9	2.0	1.6	1.0	1.4	1.3	1.0	1.4 ± 0.2
	S	3.3	3.5	3.5	3.4	3.0	3.2	3.5	3.3 ± 0.07
20 Cu	Cu	27.4	23.3	32.4	24.1	21.8	21.3	23.9	24.8 ± 1.4
	Mo	10.1	12.2	12.4	13.4	9.7	12.6	16.9	12.4 ± 0.9
	Cu:Mo	2.7	1.9	2.6	1.8	2.2	1.7	1.4	2.0 ± 0.2
	S	3.2	3.0	2.8	3.5	3.3	3.0	3.5	3.2 ± 0.1

[a]The pelleted diet used for Cu and Mo absorption trial.

[b]Cu and Mo contamination of the Control diet for mixes 2,3 and 5 occurred at the feed mill as one batch of feed for another experiment containing 100 mg Cu/kg and 50 mg Mo/kg, was made just before the diets in this experiment were mixed.

digestion. The remaining serum was stored in a seven ml acid washed test tube at -20°C for subsequent serum CpOx, Mo and TCAS Cu analysis.

Liver samples were obtained by the biopsy technique described by Chapman et al. (1963) with minor modifications as reported by Ruston (1983). Liver samples were transferred into previously acid washed, weighed and labelled six dram screw cap vials. The vials were reweighed the same day of collection to determine the weight of the liver sample before immediate wet ash digestion in the same vial.

Total urine and feces were collected from the representatives of each diet treatment, for five days during weeks 4, 8, 12 and 16 of the trial (refer to Fig. 2). Urine was collected into acid washed containers connected to the urine trays of the metabolism crates. Clean plastic sheets were placed on the urine trays to minimize contamination of urine by the tray. Feces were collected in plastic collection bags which were firmly secured to the peri-anal region of the animal by adhesive (Bull Cement, 3M Company). Feces were transferred to plastic bags every day and total fecal weight recorded for each collection period.

Analytical techniques

All glassware used in this experiment was immersed in boiling distilled water and detergent for three hrs, then washed and rinsed three times in distilled water. The glassware was then soaked in 20% HNO_3 overnight, rinsed six times with deionized distilled water and oven dried overnight at 60°C.

The liver, serum, dried feed and feces samples were wet ashed by the method of Thompson and Blanchflower (1971) with minor modifications

as follows: 1) six dram screw cap vials (vs eight drams), 2) overnight predigestion of the samples in the acid mixture (vs immediate digestion), 3) a slotted aluminum tray enclosed the lower part of the vials during digestion of the samples on a hot plate (vs slotted aluminum alloy rack) and 4) average liver, feed and feces sample weights were 0.50 g (vs liver 1.0 g and feed 0.25 g).

Ash residues from samples were dissolved with five ml of 5% HCl and were analyzed for Cu and Mo using an Instrumentation Laboratory #551 flame (acetylene and nitric oxide, respectively) atomic absorption spectrophotometer, according to the standard procedures of the Association of Official Analytical Chemists (A.O.A.C., 1980). Accuracy of this wet ashing procedure was checked by inclusion of standard biological samples obtained from U.S. Department of Commerce, National Bureau of Standards (bovine liver #1577 and orchard leaves #1571). Feed ingredient and mixed feed samples were analyzed for dry matter, gross energy, crude protein, acid detergent fibre, calcium, phosphorous, magnesium, zinc, iron and manganese (Tables 6 and 7), as described by the A.O.A.C. (1980). The total sulfur in feed was determined by the method of Boila et al. (1984).

Serum TCAS Cu was determined by the method of Mason et al. (1978b) with slight modifications. The serum sample was accurately assessed by weighing and an equal volume of 10% (w/v) TCA added. After thorough mixing, the sample was centrifuged and the supernatant (TCAS Cu) fraction was transferred to a previously acid washed and weighed seven ml test tube. The precipitate was washed by adding 1.5 ml of 5% (w/v) TCA, resuspending and recentrifuging. The washing was added to the TCAS Cu

Table 6. Average analyzed nutrient composition of pelleted diets (DM) fed to ram lambs for 16 weeks

Nutrient	Diets			
	Control	0 Cu	10 Cu	20 Cu
Dry matter (%)	91.3	91.5	91.2	91.5
Crude protein (%)	14.3	14.0	13.6	13.9
Gross energy (kJ/g)	18.4	18.3	18.2	18.2
Acid detergent fibre (%)	15.6	16.3	16.5	16.1
Calcium (%)	0.51	0.44	0.42	0.42
Phosphorous (%)	0.30	0.26	0.26	0.26
Magnesium (%)	0.20	0.20	0.20	0.20
Sulfur (g/kg)	1.8	3.3	3.3	3.2
Copper (mg/kg)	11.5[a]	7.6	16.6	24.9
Molybdenum (mg/kg)	2.8	11.2	11.9	12.5
Cu : Mo ratio	5.0	0.7	1.5	2.0
Zinc (mg/kg)	50.5	35.5	31.5	30.8
Iron (mg/kg)	135.4	86.8	86.7	82.1
Manganese (mg/kg)	18.4	17.3	16.3	16.5

[a]Control diet was contaminated as described in table 5.

Table 7. Analyzed nutrient composition of alfalfa-brome hay and barley used for pelleted diets (DM)

Nutrient	1st cut hay	2nd cut hay	barley
Dry matter (%)	89.4	89.6	91.3
Crude protein (%)	16.0	12.6	13.6
Gross energy (kJ/g)	18.4	18.4	18.5
Acid detergent fibre (%)	35.2	37.1	5.9
Calcium (%)	1.12	0.95	0.04
Phosphorous (%)	0.15	0.14	0.30
Magnesium (%)	0.30	0.24	0.13
Sulfur (g/kg)	1.6	1.4	1.4
Copper (mg/kg)	7.0	6.2	4.9
Molybdenum (mg/kg)	1.3	1.1	1.2
Zinc (mg/kg)	24.1	18.7	30.5
Iron (mg/kg)	265.2	105.4	44.5
Manganese (mg/kg)	23.1	19.5	8.7

fraction. The total weight of recovered TCAS Cu fraction was taken as the total volume of TCAS Cu and analyzed directly for Cu with a standard containing 5% TCA by flame atomic absorption spectrophotometer. The concentration of serum TCAIS Cu was obtained by calculating the differences between TCAS Cu concentration and total Cu concentration for the same serum sample. Urine Cu and Mo concentrations were determined by the method of Spector et al. (1971).

The measurement of CpOx (EC 1.16.3.1) activity in serum was based on the method of Smith and Wright (1974) as modified by Robinson (1983). Purified p-phenylene-diamine dihydrochloride (PPD) was used as a substrate in an acetate buffer (pH 6.5). The rate of PPD oxidation by CpOx was measured on a Beckman DU-8 spectrophotometer. Serum CpOx values are expressed as ΔA/min./ml of serum in this manuscript.

Statistical analysis

All data were subjected to analysis of variance using a split-plot design with repeated measurements over time by the General Linear Model (GLM) procedures, Statistical Analysis System (SAS, 1982) for unbalanced data (one animal from diet 0 Cu and one from diet 20 Cu died one day after liver biopsy in week 12). The model is:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j(\alpha_i) + \rho_k + \alpha\rho_{ik} + \varepsilon_{ijkl}$$

where:

Y = dependent variable (i.e. liver Cu)

μ = population mean

α_i = effect of the i^{th} diet

$\beta_j(\alpha_i)$ = effect of j^{th} animal within the i^{th} diet

ρ_k = effect of k^{th} time

$\alpha\rho_{ik}$ = interaction effect of the i^{th} diet and the k^{th} time

ε_{ijkl} = residual term

Animal within diet (Error A) was used to test for significance of effects of diet; and animal within diets within time (residual, Error B) was used to test for significance of sub-plot effects of time and diet x time interaction (Snedecor and Cochran, 1980). Type III sum of squares were used to provide the highest level of protection against error (SAS, 1982).

Correlations among liver, serum CpOx, TCAS Cu, TCAIS Cu and serum Mo were determined by simple linear regression, and multiple regressions were used to include diet and time effects (Snedecor and Cochran, 1980).

Duncan's multiple range test ($P<0.01$) was used to test differences among least square means using the appropriate error terms and the harmonic cell mean to adjust for unbalanced data in each diet (SAS, 1982).

RESULTS AND DISCUSSION

Liver Cu

Least square means of liver Cu concentration (Table 8) at the start of this study (week 0) were not different ($P>.05$) among diets while at the end of the study least square means were different ($P<.01$). Diet, time and diet x time interactions (Appendix Table I-1) were significantly ($P<.01$) different. Lambs on the Control diet maintained liver Cu concentration at 96.5 µg/g WB until week 4; then steadily increased to 209.5 µg/g WB at week 16 which may have been caused by accidental contamination of the diet with Cu at the feed mill. The 0 Cu diet fed lambs exhibited a decline in liver Cu reserves from 105.2 to 26.1 µg/g WB during the 16 weeks. Lambs on the 10 Cu diet exhibited a decline in liver Cu reserves from 97.2 to 57.1 µg/g WB which was maintained until week 16 of the trial. Liver Cu reserves of lambs on the 20 Cu diet declined from 112.5 to 90.3 µg/g WB at week 4; then increased to 152.6 µg/g WB at week 16 of the trial (Figure 3).

Supplemental dietary Mo and S have been used in preventing Cu toxicity or to reduce excess accumulation of Cu in the liver of sheep (Dick, 1954; Goodrich and Tillman, 1966). The addition of 10 mg Mo and 2 g S/kg to the Control diet, resulted in liver Cu concentrations of lambs on the 0 Cu diet to decrease from 105.2 to 26.1 µg/g WB (Table 8). Additions of 10 or 20 mg Cu/kg to the diet as well as Mo + S resulted in progressively higher liver Cu concentrations over the 16 weeks compared to the 0 Cu diet. This is in agreement with the results of Kline et al. (1971) who used treatments similar to the 10 Cu and 20 Cu diets of this study.

Table 8. Diet X time interaction for least square means of liver Cu (µg/g WB) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0	96.5^{Da}	105.2^{Aa}	97.2^{Aa}	112.5^{BCa}
4	96.8^{Da}	57.5^{Bb}	57.1^{Bb}	90.3^{Ca}
8	121.8^{Ca}	34.0^{Cc}	47.6^{Bc}	94.0^{Cb}
12	151.6^{Ba}	27.9^{Cc}	46.7^{Bc}	127.5^{Bb}
16	209.5^{Aa}	26.1^{Cd}	52.8^{Bc}	152.6^{Ab}

Means in the same columns (A-D) and rows (a-d) with different superscripts are significantly different ($P<.01$) using Duncan's multiple range test and SEM = 6.12

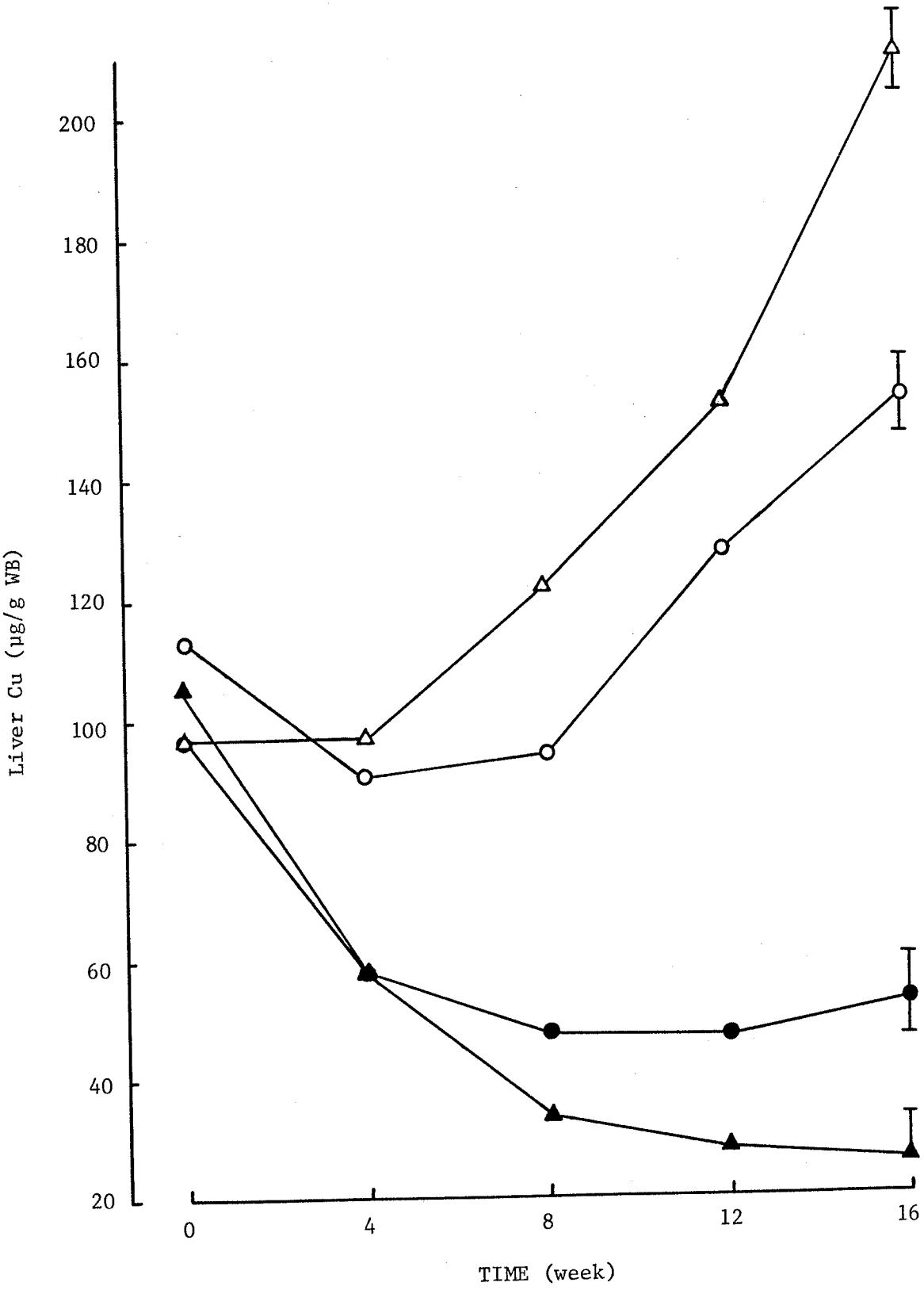


Fig. 3. Effect of supplemental Cu, Mo and S on liver Cu concentration of ram lambs fed pelleted diets for 16 weeks. Control △ ; 0 Cu ▲ ; 10 Cu ● and 20 Cu ○ .

It would appear that ram lambs fed a concentrate pelleted diet that contained approximately 11 mg Mo and 3.3 g S/kg required at least 20 mg Cu/kg to maintain adequate liver Cu reserves.

Serum Cu

Diet, time and diet x time interactions (Appendix Table I-1) were significant ($P<.01$) and diet x time least square means are shown in Figure 4. Serum Cu concentrations at the start of this study (week 0) were not different ($P>.05$) among diets but at the end of the study (week 16) the least square means were different ($P<.01$, Table 9). Serum Cu in lambs fed the Control diet fluctuated from 0.86 to 1.01 µg Cu/ml throughout the study (week 0 to week 16). Serum Cu of lambs in the 0 Cu diet increased significantly ($P<.01$) at week 4 to 1.4 µg Cu/ml and declined to 1.10 µg Cu/ml for the remainder of the trial. Lambs on the 10 Cu diet had similar serum Cu concentrations to the 20 Cu diet (Table 9).

It has been found that supplemental dietary Mo and S to a diet containing normal Cu concentration (5-7 mg Cu/kg), increased the concentration of Cu in plasma of sheep (Suttle and Field, 1968; Smith and Wright, 1975a). In the current study the serum Cu concentration of lambs fed 0 Cu diet was significantly ($P<.01$) higher than the serum Cu concentrations of the Control, 10 Cu and 20 Cu diets (Table 10). This increment of serum Cu for the 0 Cu diet was likely due to the formation of TCAIS-Cu, which has been found to accumulate in the kidney cortex and slowly excreted (Bremner and Young, 1978). However, liver Cu reserves of sheep on the 0 Cu diet were depleted, indicating that the higher

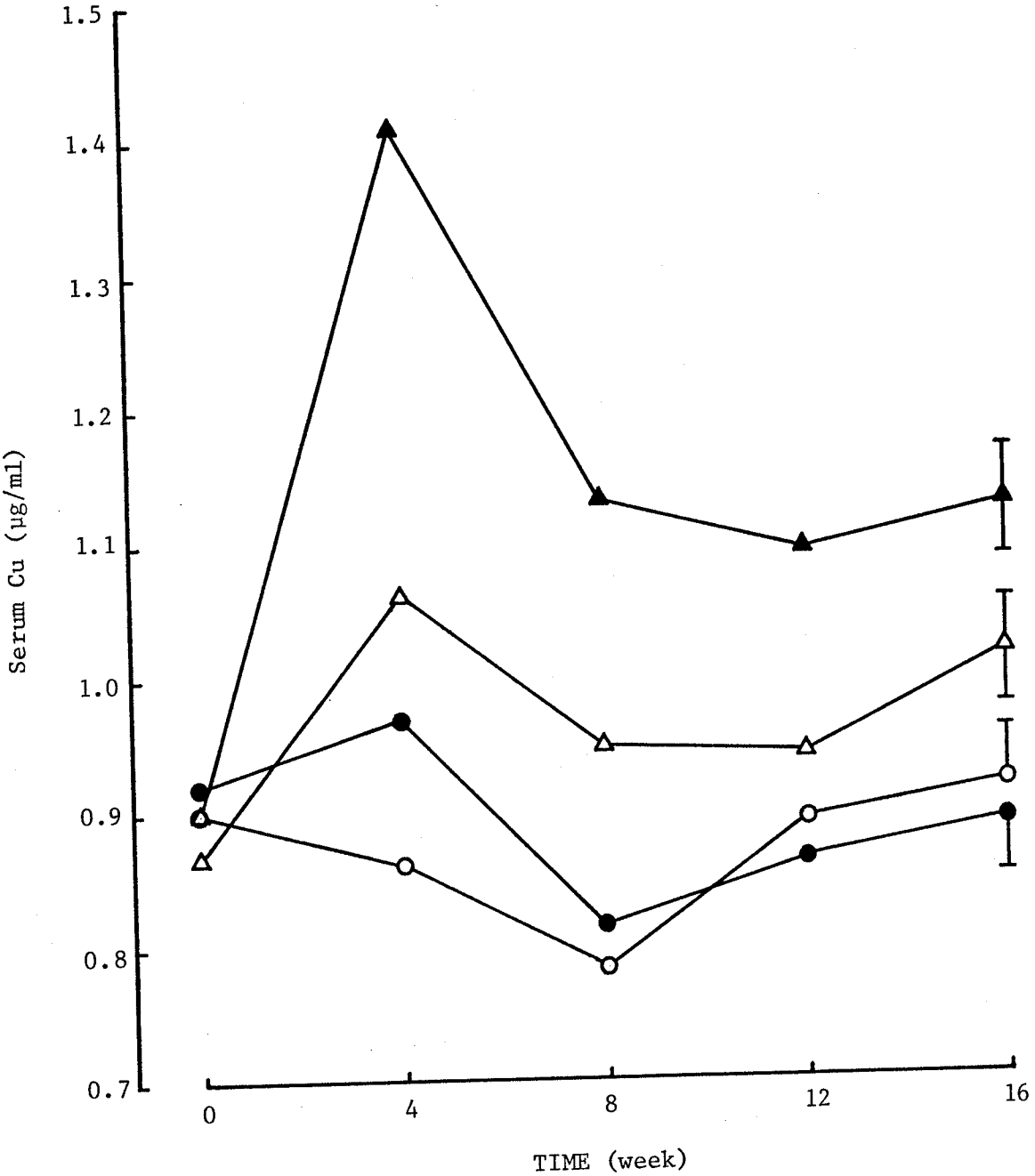


Fig. 4. Effect of supplemental Cu, Mo and S on serum Cu concentration of ram lambs fed pelleted diets for 16 weeks. Control △ ; 0 Cu ▲ ; 10 Cu ● and 20 Cu ○ .

Table 9. Diet X time interaction for least square means of serum Cu (µg/ml) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0	0.86^{Ba}	0.90^{Ca}	0.92^{Aa}	0.90^{Aa}
4	1.06^{Ab}	1.40^{Aa}	0.97^{Abc}	0.86^{Ac}
8	0.95^{ABb}	1.13^{Ba}	0.81^{Abc}	0.78^{Ac}
12	0.94^{ABab}	1.09^{Ba}	0.86^{Ab}	0.89^{Ab}
16	1.01^{ABab}	1.13^{Ba}	0.89^{Ab}	0.92^{Ab}

Means in the same column (A-C) and row (a-c) with different superscript are significantly different ($P<.01$) using Duncan's multiple range test and SEM = 0.042

Table 10. Effect of Cu, Mo and S upon overall least square means of liver Cu, serum Cu, CpOx, TCAS Cu TCAIS Cu and serum Mo of ram lambs fed pelleted diets for 16 weeks

Parameters	Diets				± MSE
	Control	0 Cu	10 Cu	20 Cu	
Liver Cu (µg/g WB)	135.2^{a}	50.1^{b}	60.3^{b}	115.4^{a}	9.18
Serum Cu (µg/ml)	0.96^{b}	1.13^{a}	0.89^{b}	0.87^{b}	0.028
CpOx (ΔA/min/ml)	0.058^{a}	0.040^{b}	0.039^{b}	0.043^{b}	0.003
Serum TCAS Cu (µg/ml)	0.85^{a}	0.71^{b}	0.72^{b}	0.74^{b}	0.024
Serum TCAIS CU (µg/ml)	0.11^{b}	0.42^{a}	0.17^{b}	0.13^{b}	0.021
Serum Mo (µg/ml)	0.17^{b}	0.54^{a}	0.19^{b}	0.13^{b}	0.033

Means in the same rows with different superscripts are significantly different ($P<.01$) using Duncan's multiple range test.

serum Cu (TCAIS-Cu) was not available for uptake by the liver. It appears that serum Cu concentration as a measure of Cu status of sheep is not reliable if Mo and S are present in excess relative to Cu in the diet. Supplemental dietary Cu to the 0 Cu diet decreased the serum Cu concentrations to a normal level as seen with the 10 Cu and 20 Cu diets (Table 10). This is in agreement with Mason et al. (1978b) who found that increasing the Cu content of diets, similar to the 0 Cu diet in this study, decreased plasma Cu to normal levels.

Serum ceruloplasmin oxidase

CpOx diet x time least square means are presented in Figure 5. Diet, time and diet x time interactions were significant ($P<.01$), Appendix Table I-2). Least square means of serum CpOx did not differ ($P>.05$) among diets at the start of the study but the least square means of CpOx were different ($P<.01$) by the end of the study (Table 11).

There have been no consistent observations reported on the CpOx activity in Mo and S supplemented animals. Suttle and Field (1968); Smith et al. (1968) and Ishida et al. (1982) have reported a decrease in CpOx activity, but Smith and Wright (1975a); Bremner (1976) and Bremner and Young (1978) were unable to demonstrate a reduction in CpOx activity in sheep fed diets containing Mo and S. In the present study, CpOx activity of diets supplemented with Mo+S were significantly ($P<.01$) reduced (Table 10).

It is not clear whether, the reduction in CpOx activity was due to failure of Cp synthesis or due to chemical inhibition by Mo in plasma of animals fed high dietary Mo and S. Lamand et al. (1980) found that

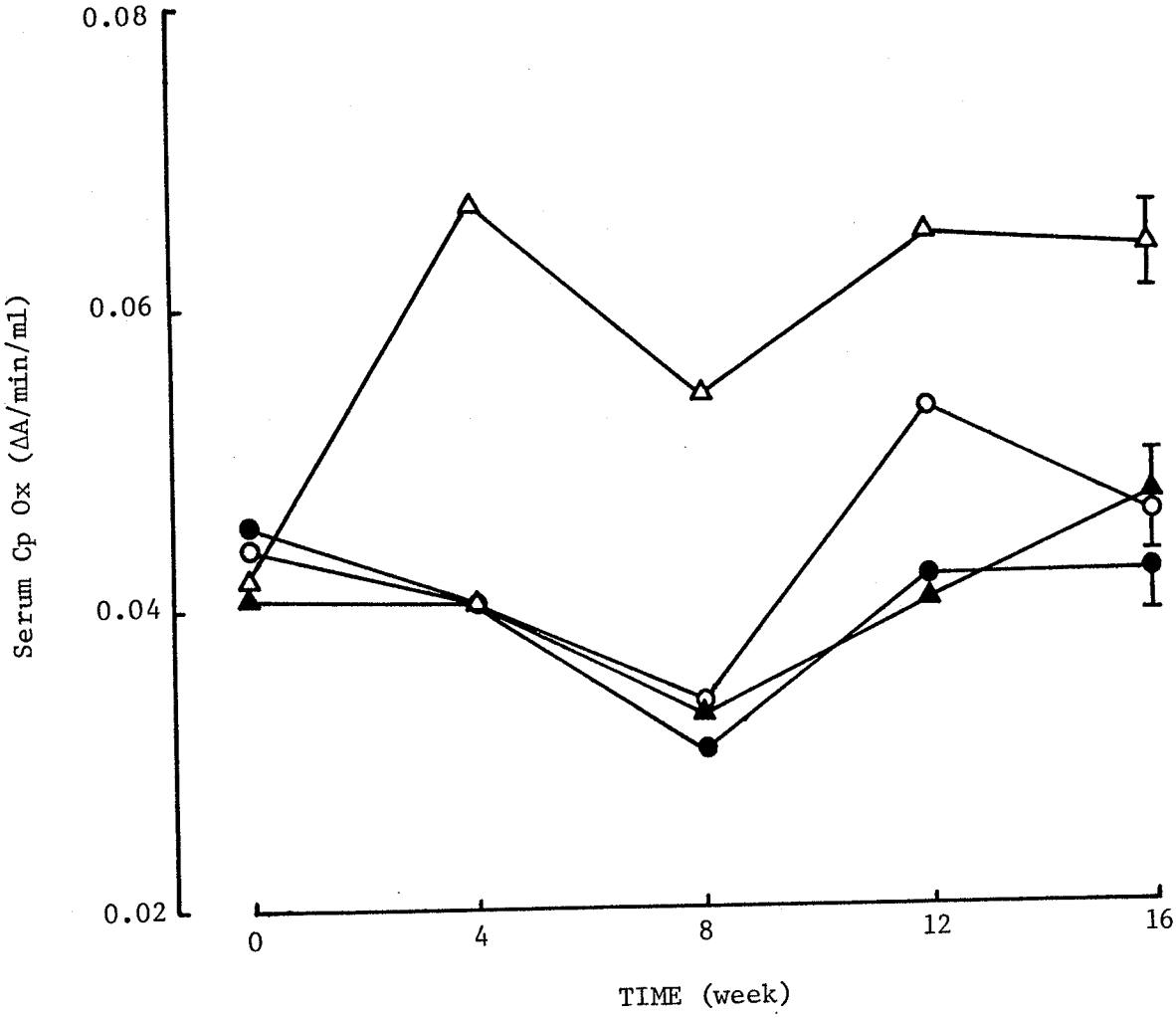


Fig. 5. Effect of supplemental Cu, Mo and S on serum Cp Ox of ram lambs fed pelleted diets for 16 weeks. Control △ ; 0 Cu ▲ ; 10 Cu ● and 20 Cu ○ .

Table 11. Diet X time interaction for least square means of CpOx (ΔA/min/ml) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0	0.042^{Ca}	0.041^{ABa}	0.045^{Aa}	0.044^{ABa}
4	0.067^{Aa}	0.040^{ABb}	0.040^{ABb}	0.040^{BCb}
8	0.054^{Ba}	0.033^{Bb}	0.030^{Bb}	0.033^{Bb}
12	0.064^{ABa}	0.040^{ABc}	0.042^{Ac}	0.053^{Ab}
16	0.063^{ABa}	0.047^{Ab}	0.042^{Ab}	0.046^{ACb}

Means in the same columns (A-C) and rows (a-c) with different superscripts are significantly different ($P<.01$) using Duncan's multiple range test and SEM = 0.003

supplementing 3 g S and 13 mg Mo/kg DM to a diet containing 3.6 mg Cu/kg significantly reduced CpOx activity, and it was concluded that the decrease was due to a failure of Cp synthesis. Mason et al. (1980) demonstrated that duodenally infused Mo labelled tetrathiomolybdate inhibited CpOx activity and they suggested that Mo in the TCAIS form caused the inhibition of CpOx activity. The results of the current study do not agree with the findings of either Lamand et al. (1980) or Mason et al. (1980) in regard to Cp synthesis or inhibition of activity by Mo in TCAIS. Liver Cu reserves in the 20 Cu diet were higher compared to the 0 Cu diet which would suggest that the Cu was available for Cp synthesis. In the case of Cp inhibition by Mo in TCAIS fractions, the Mo concentration in serum of the 20 Cu lambs was significantly lower than for lambs on the 0 Cu diet even though the CpOx activity of both 0 Cu and 20 Cu were depressed, indicating that the Mo present in the serum did not affect Cp activity. More research is required to elucidate the effect of dietary Mo and S on CpOx of sheep.

Serum TCAS-Cu and TCAIS-Cu

Diet x time interactions for least square means of serum TCAS-Cu are plotted in Figure 6. From the results of statistical analysis, diet, time and diet x time interactions were significant ($P<.01$, Appendix Table I-2). Least square means of serum TCAS-Cu were not different ($P>.05$) among diets at the start of this study but they were different ($P<.01$) at the end of the study (Table 12). Serum TCAS-Cu concentrations in Control lambs were significantly ($P<.01$) higher than supplemented

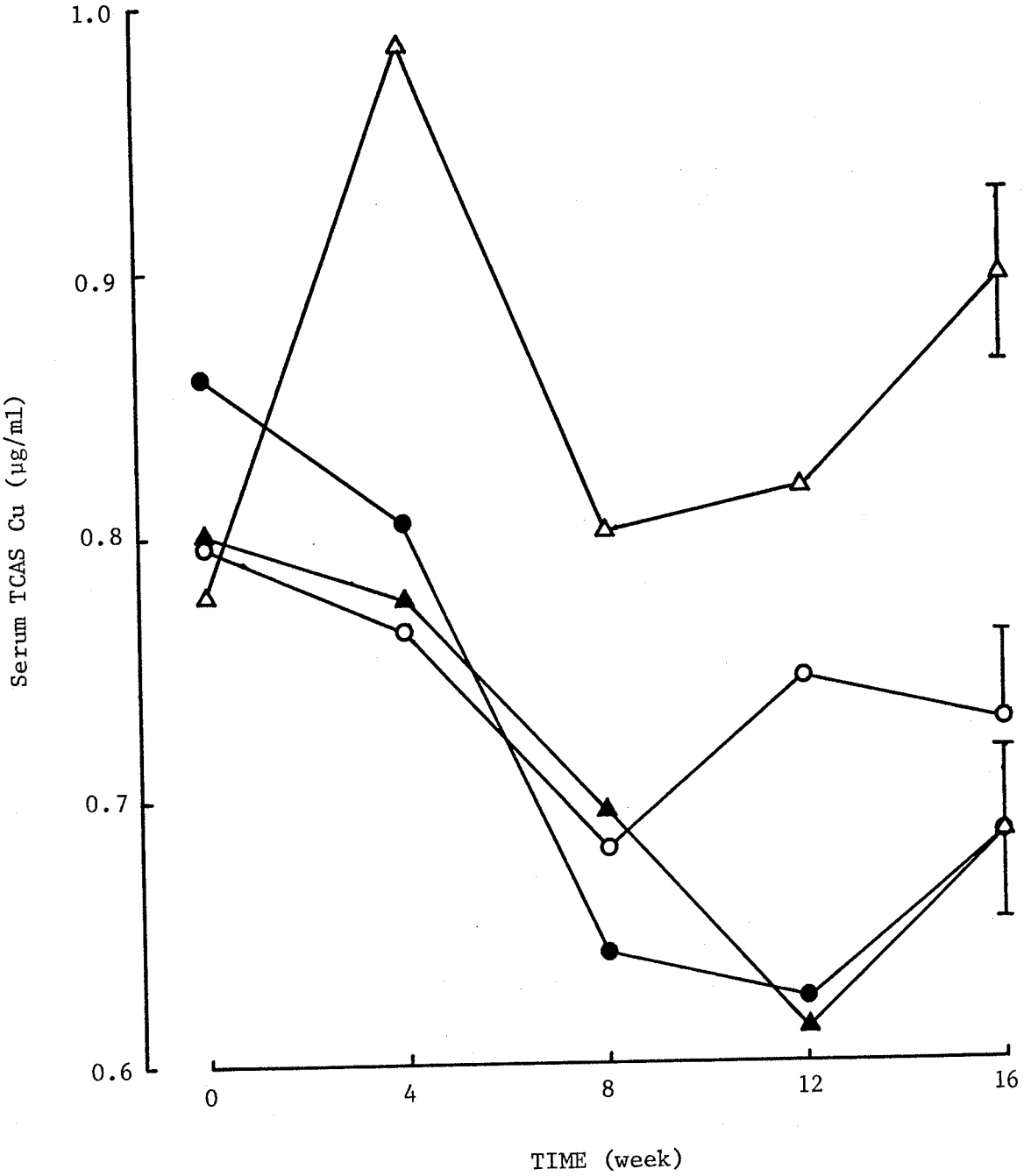


Fig. 6. Effect of supplemental Cu, Mo and S on serum TCAS Cu concentration of ram lambs fed pelleted diets for 16 weeks. Control △ ; 0 Cu ▲ ; 10 Cu ● and 20 Cu ○ .

Table 12. Diet X time interaction for least square means of serum TCAS Cu (µg/ml) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0	0.78^{Ba}	0.80^{Aa}	0.86^{Aa}	0.79^{Aa}
4	0.98^{Aa}	0.77^{Ab}	0.80^{ACb}	0.76^{Ab}
8	0.80^{Ba}	0.69^{ABab}	0.64^{Bb}	0.68^{Aab}
12	0.81^{Ba}	0.61^{Bc}	0.62^{Bbc}	0.74^{Aab}
16	0.89^{ABa}	0.68^{ABb}	0.68^{BCb}	0.73^{Ab}

Means in the same columns (A-C) and rows (a-c) with different superscript are significantly different ($P<.01$) using Duncan's multiple range test and SEM = 0.033

lambs and there were no significant differences for TCAS-Cu among the 0 Cu, 10 Cu and 20 Cu diet fed lambs (Table 10). In contrast to TCAS-Cu, serum TCAIS-Cu of lambs fed the 0 Cu diet was higher ($P<.01$) than the Control, 10 Cu and 20 Cu diet fed lambs and there were no differences ($P>.01$) for TCAIS-Cu of lambs fed the Control, 10 Cu and 20 Cu diets (Table 10).

Diet, time and diet x time interactions of serum TCAIS-Cu were significant ($P<.01$, Appendix Table I-2). Diet x time interaction for least square means of TCAIS-Cu are presented in Figure 7. Least square means of serum TCAIS-Cu concentrations were not different ($P>.05$) among diets at the start of the study but at the end of the study they were different ($P<.01$, Table 13). TCAIS-Cu for lambs fed the 0 Cu diet increased significantly ($P<.01$) from 0.10 to 0.63 at week 4 and then declined to 0.45 µg Cu/ml for the rest of the study.

Serum TCAIS-Cu for lambs fed the 0 Cu diet had the same pattern as serum Cu, where both these parameters were higher ($P<.01$) than Control, 10 Cu and 20 Cu diet fed lambs (Table 10). There was a high positive correlation between serum Cu and TCAIS-Cu ($r = +0.87$) only with the 0 Cu diet fed lambs (Table 14). However, serum Cu was correlated with TCAS-Cu with r values of +0.92, +0.79 and +0.84 for the Control, 10 Cu and 20 Cu diet fed lambs, respectively. This indicates that if the Cu:Mo ratio in a diet is low, serum TCAIS-Cu is a better indicator of serum Cu, but if the ratio is high, serum TCAS-Cu is a better indicator of serum Cu.

It has been generally accepted that the Cu which is bound loosely with albumin (direct reacting, Dr-Cu) is an immediate transport form of

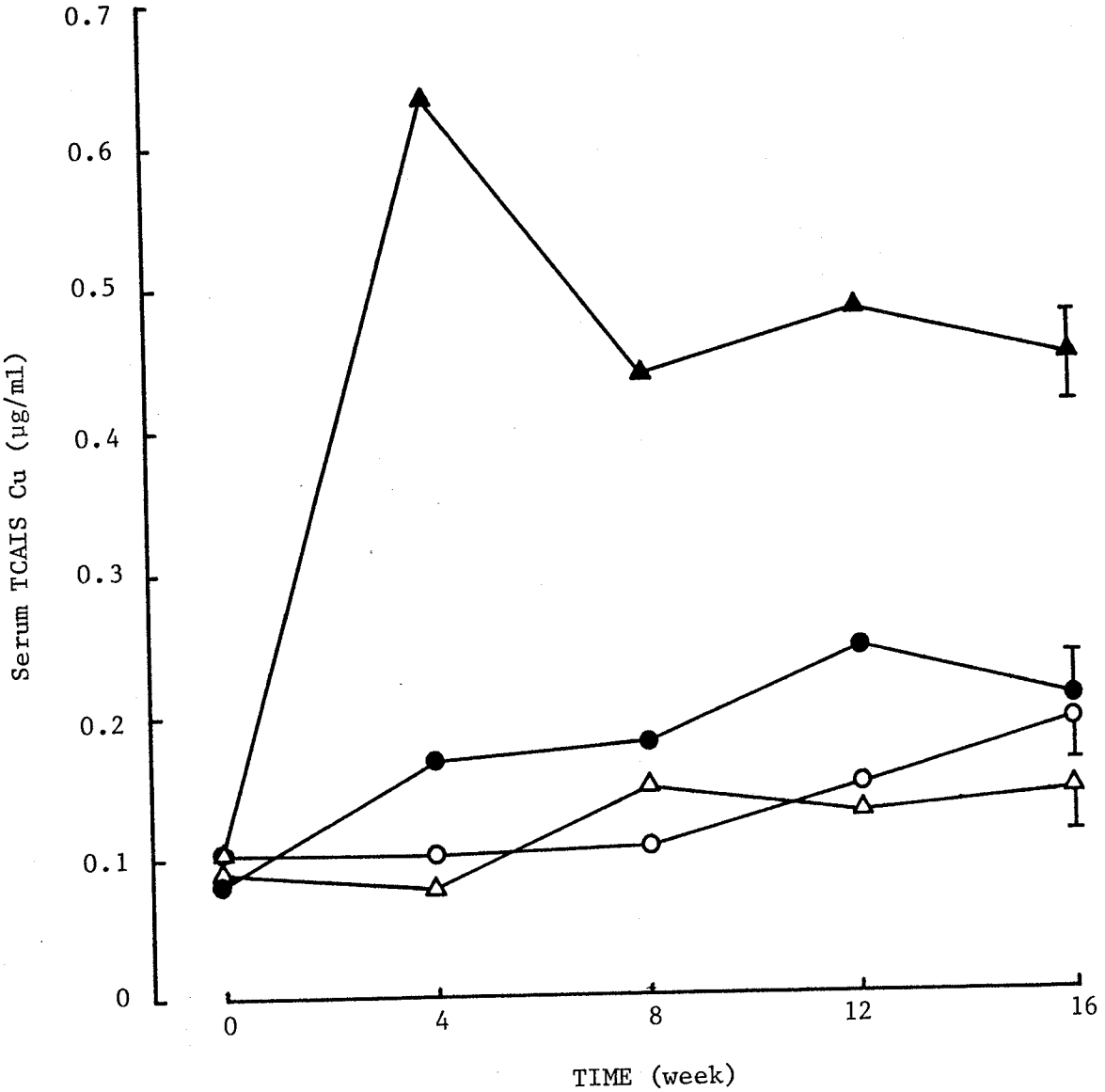


Fig. 7. Effect of supplemental Cu, Mo and S on serum TCAIS Cu concentration of ram lambs fed pelleted diets for 16 weeks. Control △ ; 0 Cu ▲ ; 10 Cu ● and 20 Cu ○ .

Table 13. Diet X time interaction for least square means of serum TCAIS Cu (μg/ml) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0	0.09^{Aa}	0.10^{Ca}	0.08^{Ba}	0.10^{Aa}
4	0.07^{Ab}	0.63^{Aa}	0.17^{ABb}	0.10^{Ab}
8	0.15^{Ab}	0.43^{Ba}	0.17^{ABb}	0.10^{Ab}
12	0.13^{Ab}	0.48^{Ba}	0.24^{Ab}	0.15^{Ab}
16	0.14^{Ab}	0.45^{Ba}	0.20^{Ab}	0.19^{Ab}

Means in the same columns (A-C) and rows (a-b) with different superscripts are significantly different ($P<.01$) using Duncan's multiple range test and SEM = 0.031

Table 14. Correlation coefficients (r) between parameters measured for ram lambs fed pelleted diets for 16 weeks

	Serum Cu	CpOx	TCAS Cu	TCAIS Cu	Serum Mo[1]
Liver Cu	(-0.06)[2]	(0.52)	(0.47)	(-0.41)	(-0.52)
Control	0.16	0.34	0.02	0.42	-0.21
0 Cu	-0.20	-0.04	0.48	-0.43	0.48
10 Cu	0.23	0.35	0.53	-0.50	-0.13
20 Cu	0.67	0.60	0.55	0.32	-0.50
Serum Cu		(0.28)	(0.40)	(0.73)	(0.56)
Control		0.81	0.92	0.21	0.20
0 Cu		-0.07	0.14	0.87	0.78
10 Cu		0.57	0.79	0.22	-0.12
20 Cu		0.77	0.84	0.44	-0.37
CpOx			(0.72)	(-0.24)	(-0.35)
Control			0.70	0.26	-0.28
0 Cu			0.51	-0.32	-0.63
10 Cu			0.61	-0.12	-0.18
20 Cu			0.73	0.19	-0.36
TCAS Cu				(-0.33)	(-0.26)
Control				-0.20	0.40
0 Cu				-0.37	-0.40
10 Cu				-0.42	-0.33
20 Cu				-0.11	-0.15
TCAIS Cu					(0.78)
Control					-0.33
0 Cu					0.87
10 Cu					0.29
20 Cu					-0.53

[1]Serum Mo concentrations were available for only the last three collection periods, therefore the correlations between serum Mo and other parameters were obtained for these periods (week 8, 12 and 16).

[2]The values in parentheses indicate the overall correlation coefficients between two parameters.

Cu in plasma, and is increased by dietary supplementation of Mo+S. This has been observed in all experiments reported thus far. However, the results of the present study indicate that the dietary supplementation of Mo and S (0 Cu diet) had such adverse effects on Cu utilization as decreases in TCAS-Cu, CpOx activity and liver Cu reserves (Table 10). The fact that the concentration of serum TCAIS-Cu of lambs fed 0 Cu diet was higher than Control lambs after the administration of Mo and S, indicate that the increase of serum Cu of 0 Cu diet fed lambs was due to TCAIS-Cu which was not available for tissue uptake.

Dick et al. (1975) reported that the complex of Mo and S, thiomolybdate (TM), produced TCAIS in plasma which could be involved in the mechanism for the formation of unavailable Cu in animals fed Mo and S. This hypothesis has been supported by investigations providing additional evidence that TM results in increased plasma TCAIS-Cu in sheep (El-Galled et al., 1977), decreased CpOx activity and decreased intestinal ^{64}Cu absorption and liver Cu in rats (Mills et al., 1978). The results of the present study would also support this TM hypothesis.

Serum Mo

Diet x time interaction for least square means of serum Mo are presented in Table 15. Serum samples for serum Mo analysis were not available at 0 and 4 weeks, therefore serum Mo concentrations among diets cannot be compared at 0 and 4 weeks. Diet ($P<.01$), time ($P<.01$) and diet x time ($P<.05$) interactions were statistically significant (Appendix Table I-2). The increase in serum Mo concentration of lambs fed the

Table 15. Diet X time interaction for least square means of serum Mo (μg/ml) of ram lambs fed pelleted diets for 16 weeks[#]

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
8	1.27*	0.60^{Aa}	0.18^{Ab}	0.24^{Ab}
12	0.16^{Ab}	0.51^{Aa}	0.24^{Ab}	0.13^{ABb}
16	0.19^{Ab}	0.50^{Aa}	0.16^{Ab}	0.02^{Bc}

[#]Serum for Mo analysis was available only the last three collection periods (weeks 8, 12 and 16).

*The increment of serum Mo in the control diet (week 8) has been caused by the contaminated diet and not been included in this comparison test.

Means in the same columns (A-B) and rows (a-c) with different superscripts are significantly different ($P<.01$) using Duncan's multiple range test and SEM = 0.036

Control diet at week 8, was thought to be caused by contamination of the diet at the feed mill (refer to Table 5). Serum Mo concentration in the 0 Cu diet fed lambs was significantly ($P<.01$) higher than the Control, 10 Cu and 20 Cu diet fed lambs (Table 10). There were high correlations between serum Mo and serum Cu ($r = +0.78$), and serum Mo and serum TCAIS-Cu ($r = +0.87$) only for the 0 Cu diet (Table 14). These correlations among serum Cu, Mo and TCAIS-Cu indicated that the increase of serum Cu for the 0 Cu diet was caused by the TCAIS fraction of serum which contained TCAIS-Cu and Mo. These results would confirm the report of Mills et al. (1978) who suggested that the elevated serum Cu was associated with high serum TCAIS-Cu and Mo concentrations in diets similar to the 0 Cu diet in this study.

Body weight and feed intake

The mean body weight of lambs at the start of the study for all diets was 34.7±6.6 kg and after 16 weeks the body weights were 65.5, 65.5, 64.5 and 67.3 kg ($P>.05$) for the Control, 0 Cu, 10 Cu and 20 Cu diets respectively. Diet and diet x time interactions were not significant ($P>.05$), but time differences were statistically significant ($P<.01$, Appendix Table I-1). Diet x time least square means of body weights are presented in Table 16. There were no significant differences ($P>.05$) among mean body weights, but lambs on the 20 Cu diet had a tendency to heavier body weights than lambs on the other diets (Table 16).

There was no significant difference ($P>.05$) in feed intake among diets (Table 17). Feed intake increased over the first 12 weeks of the

Table 16. Diet X time interaction for least square means of body weight (Kg) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0	34.6^{E}	34.7^{E}	34.9^{D}	34.7^{E}
4	42.3^{D}	43.1^{D}	43.6^{C}	42.4^{D}
8	51.7^{C}	52.2^{C}	52.2^{B}	54.9^{C}
12	60.6^{B}	62.0^{B}	61.3^{A}	63.4^{B}
16	65.5^{A}	65.5^{A}	64.5^{A}	67.3^{A}

Means in the same columns (A-E) with different superscripts are significantly different ($P<.01$) using Duncan's multiple range test and MSE = 0.92 .

Table 17. Daily feed consumption (Kg,DM) of ram lambs fed pelleted diets for 16 weeks

Week	Diets			
	Control	0 Cu	10 Cu	20 Cu
0 - 4	1.90	1.80	1.69	1.85
4 - 8	2.07	2.04	2.06	2.19
8 - 12	2.16	2.22	2.11	2.28
12 - 16	1.91	1.87	1.75	1.67

study and declined during the last 4 weeks. A possible explanation for the decline in feed intake may be related to a change in environment, as the lambs were moved to enclosed pens equipped with a heating system ten weeks after the start of the study.

Apparent absorption and urinary excretion of Cu and Mo

The apparent absorptions of Cu and Mo over time are presented in Figure 8. The apparent absorption of Cu in the Control lamb was higher than in the supplemented lambs, whereas the absorption of Cu in the 0 Cu fed lamb was the lowest (Table 18). In contrast to Cu absorption, the apparent absorption of Mo by the 0 Cu lamb was higher than the Control, 10 Cu and 20 Cu diet fed lambs. The data were not subjected to statistical analysis, as only one lamb was used (limited facility) for each diet for the digestion and absorption trial. The quantities and concentrations of Cu and Mo in the diets, feces and urine used for the calculation of absorption (%) and urinary output of Cu and Mo are presented in Appendix II.

The Cu absorption patterns (Figure 8) were similar to the pattern for liver Cu concentrations (Figure 3) among all diets. This would indicate that as more Cu was absorbed from the digestive tract, more Cu was stored in the liver.

Dick et al. (1975) suggested that the TM which is formed in the rumen combines with Cu and is not available for absorption. The TM that does not combine with rumen Cu is absorbed into the blood stream and mobilizes tissue or reserve Cu. The results of this study would support

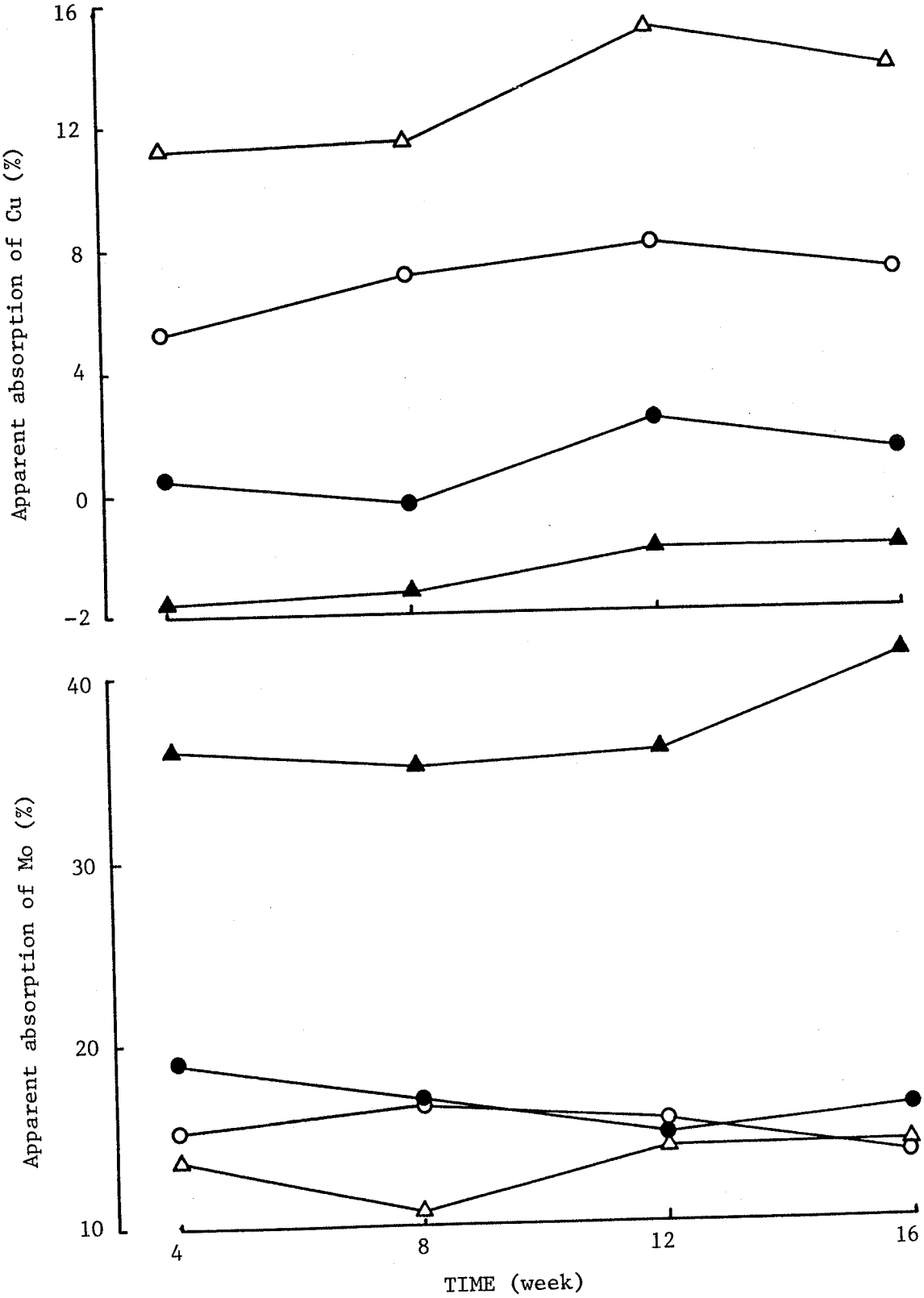


Fig. 8. Effect of supplemental Cu, Mo and S on apparent absorption of Cu and Mo of ram lambs fed pelleted diets for 16 weeks. Control △ ,0 Cu ▲ ,10 Cu ● and 20 Cu ○ .

Table 18. Effect of supplemental Cu, Mo and S upon mean (MSE) of apparent absorption (%) and urinary output (mg/5days) of Cu and Mo by ram lambs fed pelleted diets for 16 weeks

Parameters	Diets			
	Control	0 Cu	10 Cu	20 Cu
Apparent absorption of Cu	12.8(0.91)	-2.6(0.47)	0.9(0.57)	6.7(0.54)
Apparent absorption of Mo	13.2(0.90)	36.8(1.35)	16.6(0.91)	15.4(0.52)
Urinary output of Cu	0.5(0.04)	0.8(0.07)	0.6(0.04)	0.5(0.08)
Urinary output of Mo	2.7(0.65)	26.6(2.30)	15.9(1.61)	12.5(2.10)

this hypothesis, as the lambs fed the 0 Cu diet had lower Cu absorption and higher Mo absorption than lambs fed the Control diet. However, the results indicate that the absorptions of Cu and Mo depend upon the quantities of Cu and Mo present in the diet. This suggests that if Cu:Mo ratio in a diet was low, Cu absorption was impaired but Mo absorption was enhanced. By increasing the Cu:Mo ratio in the diets (0 Cu vs 10 and 20 Cu) the Cu absorption increases and Mo absorption decreases.

Urinary outputs of Cu and Mo over time are presented in Figure 9. Lambs fed the 0 Cu diet had higher urinary Cu and Mo concentrations than the lambs fed the Control, 10 Cu and 20 Cu diets. This increase of Cu and Mo in the urine of the 0 Cu lambs was possibly due to the formation of a CuMo complex in the serum, as a result of an interaction of absorbed TM (Mo) with blood Cu (from liver), which was unavailable to the animal and was excreted in the urine. Bremner and Young (1978) found that the same CuMo complex in plasma also occurred in the kidney cortex and was excreted in the urine.

The urine volume and percent fecal dry matter for lambs fed the Control and the supplemented diets were different. The lamb fed the 0 Cu diet had a higher fecal dry matter and urine volume than lambs fed the Control, 10 Cu and 20 Cu diets (Table 19). These values were progressively smaller as the Cu:Mo ratio in the diets increased (0 Cu lamb vs 10 Cu and 20 Cu lambs). Marcilese et al. (1970) reported that the addition of Mo and inorganic sulfate to the diet of sheep resulted in greater accumulation of Cu in the kidney and increased excretion of Cu in urine. They also found that urine volume was greatly increased if Mo and inorganic sulfate were added to the diet. The results of the

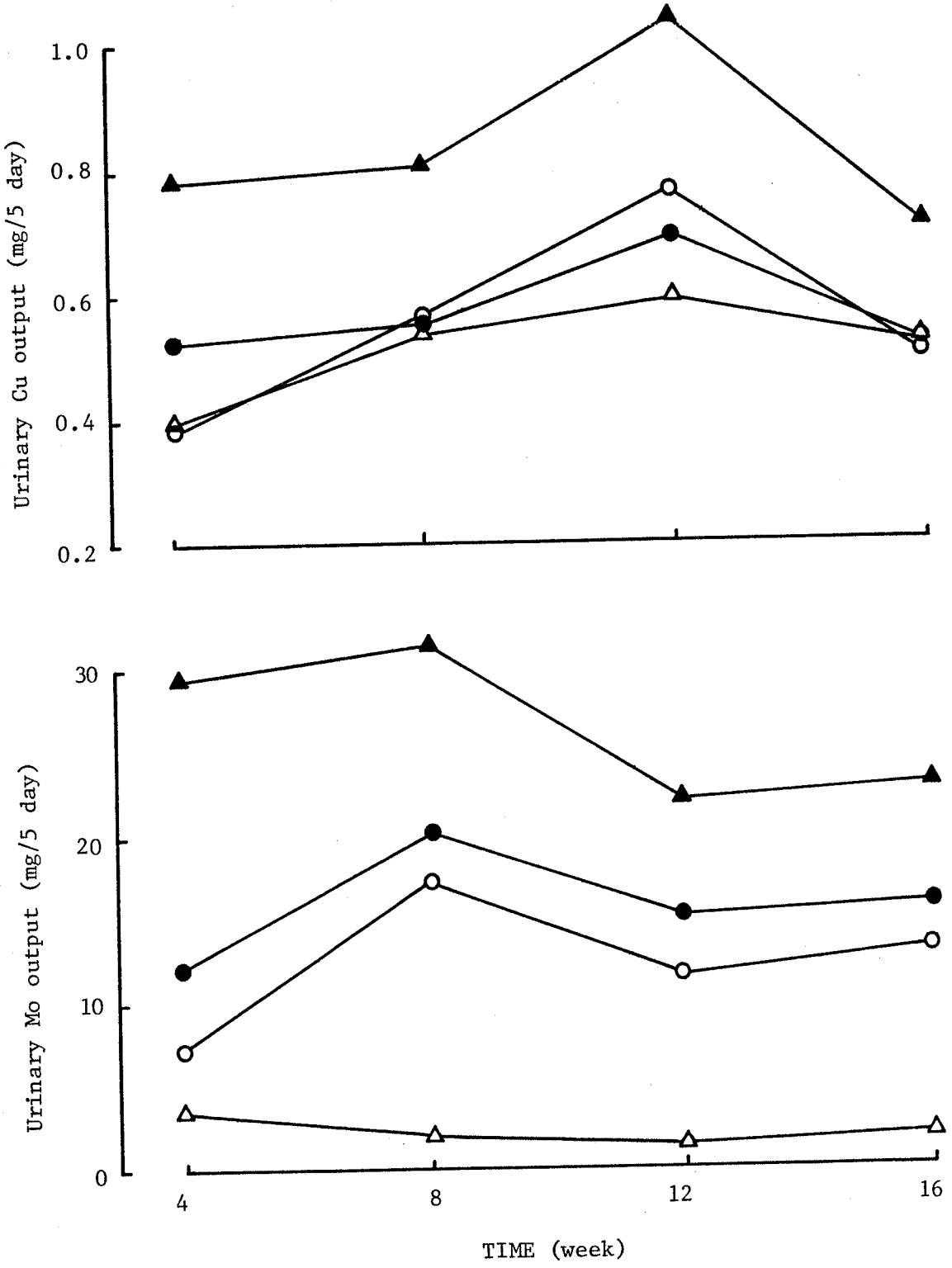


Fig. 9. Effecrt of supplemental Cu, Mo and S on urinary excretion of Cu and Mo of ram lambs fed pelleted diets for 16 weeks. Control △ ; 0 Cu ▲ ; 10 Cu ● and 20 Cu ○ .

Table 19. Effect of supplemental Cu, Mo and S on feces dry matter and urinary output of ram lambs fed pelleted diets during 5 days in each collection period (week 4, 8, 12 and 16)

		Diets			
		Control	0 Cu	10 Cu	20 Cu
Feces dry matter (%)					
Week	4	27.13	31.11	31.29	28.68
	8	23.64	31.34	27.43	26.62
	12	25.92	35.57	31.04	28.36
	16	28.18	35.15	29.64	31.64
Mean	$\bar{X}$	26.18	33.29	29.85	28.82
	SE	0.95	1.19	0.88	1.04
Urine output (liter)					
Week	4	5.48	7.65	5.98	5.20
	8	5.75	7.53	6.01	5.76
	12	5.20	7.37	6.00	5.49
	16	5.49	6.48	5.47	4.29
Mean	$\bar{X}$	5.48	7.26	5.87	5.18
	SE	0.112	0.265	0.131	0.320

present study confirmed the results of Marcilese et al. (1970) but only with the lambs fed the 0 Cu diet. However, the urine volumes and fecal dry matters of lambs fed 10 Cu and 20 Cu diets progressively declined with the addition of Cu to the diet.

Cu, Mo and S interactions

The overall least square means of liver Cu and serum parameters are presented in Figure 10. To facilitate in interpretation of the results of the present study a model was designed to describe the effects of Mo and S on Cu metabolism in sheep (Figure 11).

High levels of dietary S either as sulfate or as sulfur amino acids, would increase the ruminal sulfide ($S^{=}$) production, and consequently increase the tendency of molybdate to react with sulfide to form TM (reaction 1). There follows two possibilities, absorption of TM or Cu, the predominant route would be mainly dependent upon the relative amounts of available TM and Cu. In any case, TM interacts with Cu in the rumen to form an insoluble CuTM complex (reaction 2) and also, some amount of Cu interacts with the sulfide present in the rumen to form the insoluble Cu-S (reaction 3). As both CuTM and Cu-S are insoluble products they would be passed through the intestines and excreted in feces (Figure 11).

A TM excess relative to Cu in the rumen, would result in increased absorption of TM (Mo) and decreased absorption of Cu. Dick et al. (1975) envisaged the interactions of absorbed TM with blood Cu (derived from liver Cu pool) to form metabolically unavailable CuTM complexes (reaction 4) i.e. the TCAIS-Cu fraction in the blood.

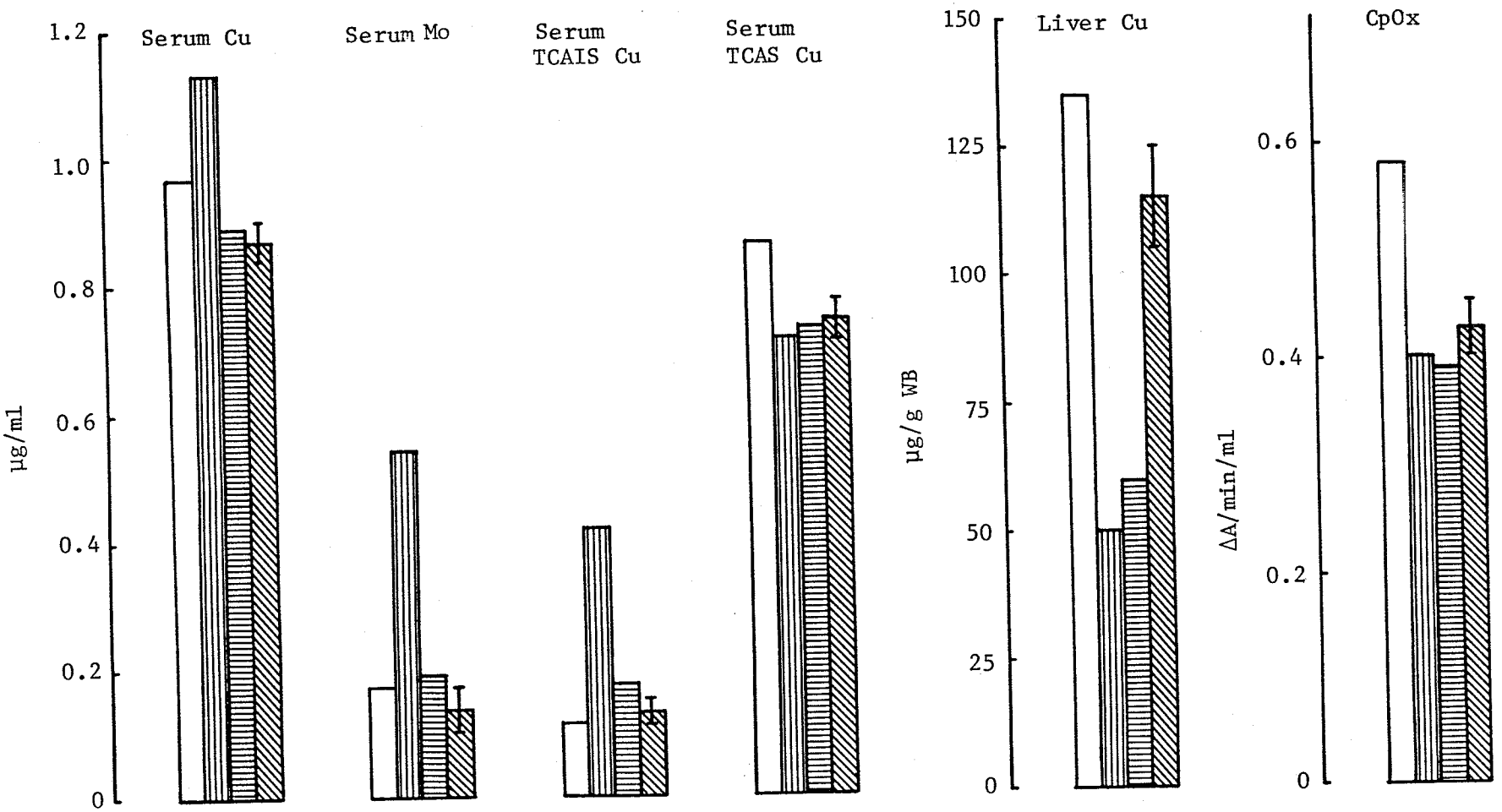
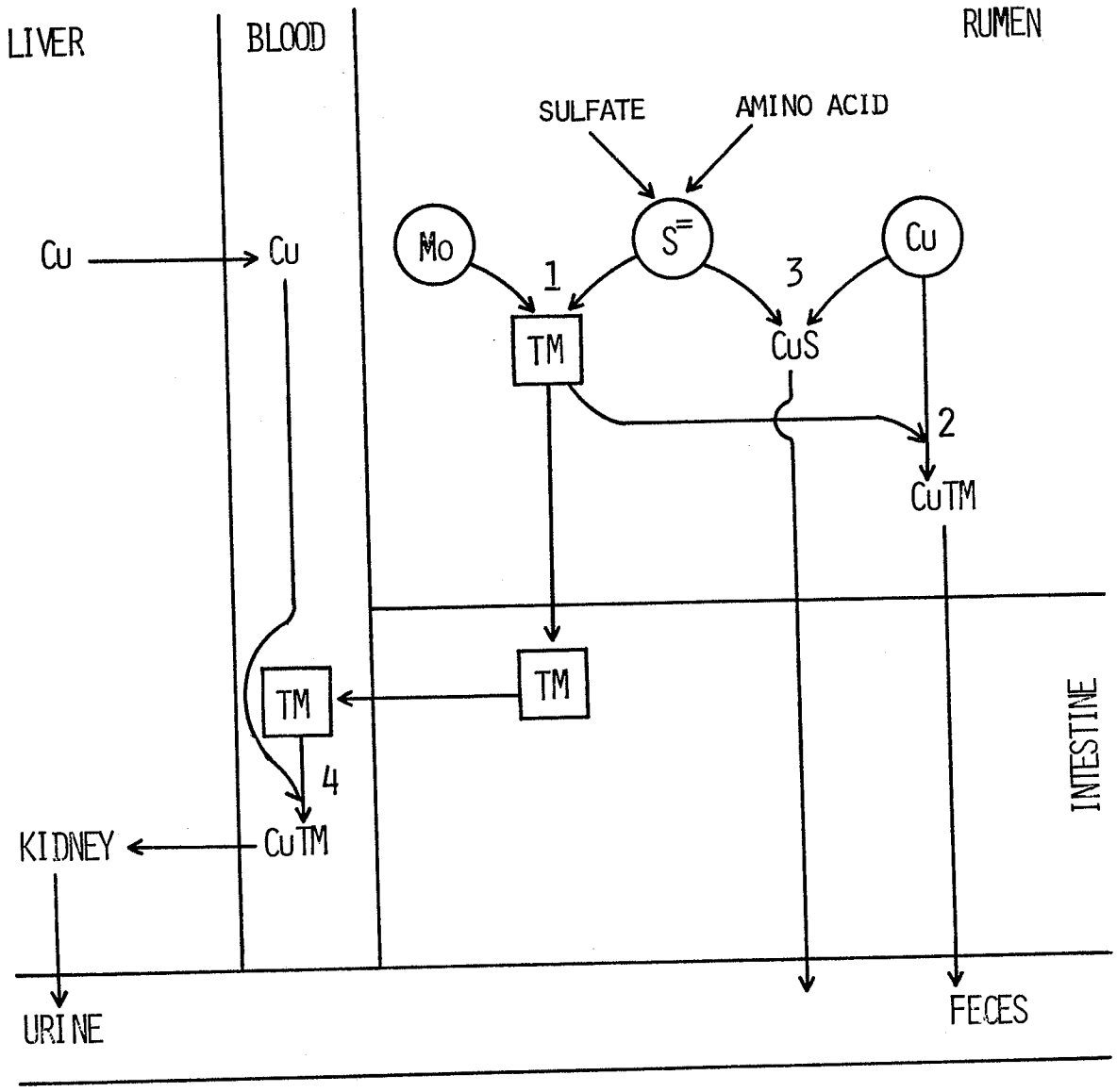


Fig. 10. Effect of supplemental Cu, Mo and S upon overall least square means of liver Cu and serum parameters of ram lambs fed pelleted diets for 16 weeks, ☐ Control; |||| 0 Cu; ≡ 10 Cu; ▨ 20 Cu.



Result:

- ↑ Serum Cu, Mo, TCAIS Cu
- ↑ Mo absorption
- ↑ Urine Cu, Mo
- ↓ Serum CpOx, TCAS Cu
- ↓ Cu absorption
- ↓ Liver Cu

Fig. 11. A model for mechanism involved in the inhibitory action of dietary Mo and S upon Cu utilization of ram lambs fed 0 Cu diet (Cu:Mo ratio of 0.7 and 3.3 g S/kg).

The results observed with the lambs fed the 0 Cu diet, with a Cu:Mo ratio of 0.7 and 3.3 g S/kg, would substantiate this hypothesis since absorption of Mo was clearly increased, Cu absorption decreased (Table 18) and liver Cu reserves depleted (Figure 10) indicating that the absorbed TM (Mo) combined with tissue Cu. Consequently, serum total Cu and Mo and TCAIS-Cu increased indicating the formation of the unavailable CuTM complex which resulted in an increased urinary excretion of Cu and Mo (Table 18).

A Cu excess in the diet, which was the case with lambs fed to the 20 Cu diet, with a Cu:Mo ratio of 2.0 and 3.3. g S/kg, resulted in increased absorption of Cu and decreased Mo absorption compared to the lamb fed the 0 Cu diet (Table 18). Subsequently, liver Cu reserves increased and serum total Cu, Mo and TCAIS-Cu concentrations decreased (Figure 10). Therefore, the urinary output of Cu and Mo with the lamb fed the 20 Cu diet decreased in comparison to the lamb fed the 0 Cu diet, and less TM (Mo) was absorbed, thus the systemic effect on Cu utilization in the 20 Cu diet fed lamb did not occur. The results with the 20 Cu diet were similar to those observed with the Control diet (Figure 10).

In the case of the 10 Cu diet, with a Cu:Mo ratio of 1.5 and 3.3 g S/kg, the amount of Cu in the diet was not sufficient to react with TM in the rumen to form the unavailable CuTM complex. Therefore, the absorption of Mo and consequently the concentrations of serum Cu, Mo and TCAIS-Cu (Figure 10) and urinary output of Cu and Mo (Table 18) were slightly higher than in the 20 Cu diet, even though these parameters were not statistically different between the two diets (10 Cu vs 20 Cu).

The results described herein are comparable with the hypothesis outlined by Dick et al. (1975) which suggests ruminal TM formation and subsequent reaction of TM with Cu in the rumen to form unabsorbable Cu complexes. The quantity of TM that was not in combination with Cu in the rumen was absorbed into the blood and mobilized tissue Cu, resulting in elevated blood Cu levels. Thus the formation of TCAIS-Cu would play an important role in rendering Cu metabolically unavailable. More work however, will be necessary before the hypothesis of the role of TM will be totally accepted.

SUMMARY AND CONCLUSIONS

1. The liver Cu concentration of lambs was significantly ($P<.01$) affected by diet and diet x time interaction. Liver Cu levels of lambs decreased when fed a diet with a Cu:Mo ratio of less than 2.0 with 3.3 g S/kg, while increasing the Cu:Mo ratio to above 2.0 in the diet, resulted in increased liver Cu levels.
2. The enzyme activity of serum CpOx and serum TCAS-Cu concentration of lambs were significantly ($P<.01$) affected by diet and diet x time interaction. The lambs on the Control diet had higher CpOx and TCAS-Cu than supplemented lambs. The correlation ($r = +0.72$) between these two parameters was significant ($P<.01$) which indicated that, the serum TCAS-Cu as an independent variable increased, CpOx as a dependent variable also increased.
3. The concentrations of serum Cu, TCAIS Cu and Mo of lambs were significantly ($P<.01$) affected by diet. The lambs fed 0 Cu diet had higher serum Cu, TCAIS-Cu and Mo than the lambs fed the Control, 10 Cu and 20 Cu diets. There were significant ($P<.01$) positive correlations between serum Cu and TCAIS Cu ($r = +0.87$), serum Cu and serum Mo ($r = +0.78$) and TCAIS-Cu and serum Mo ($r = +0.87$) in the 0 Cu diet. This indicated that as serum TCAIS-Cu and Mo as an independent variable increased, serum Cu as a dependent variable increased, which suggested that the increased serum Cu was a result of TCAIS-Cu and Mo in the serum.
4. Lambs on the 0 Cu diet had higher serum Cu, Mo and TCAIS-Cu, but lower liver Cu compared to the lambs fed the Control diet. By increasing the dietary Cu:Mo ratio (0 Cu vs 20 Cu) resulted in a

lower serum Cu, Mo and TCAIS-Cu and a higher liver Cu level.

5. There were no significant differences ($P>.05$) among mean body weights and feed intakes of lambs fed supplemental dietary Cu, Mo and S for 16 weeks.

6. The apparent absorption of Cu in lambs fed the Control diet was higher than supplemented lambs, whereas the apparent absorption of Mo for the lamb on the 0 Cu diet was higher than the Control, 10 Cu and 20 Cu diet fed lambs. This indicated that the absorption of Cu and Mo depends upon the quantities of Cu and Mo present in the diet. It would suggest that if the Cu:Mo ratio in a diet was low, Cu absorption was impaired but Mo absorption was enhanced. Increasing the Cu:Mo ratio in the diet (0 Cu vs 20 Cu) the Cu absorption increased and Mo absorption decreased.

7. The lamb on the 0 Cu diet had a higher urinary Cu and Mo concentration than the lambs fed the Control, 10 Cu and 20 Cu diets. This increase of Cu and Mo in the urine of the 0 Cu diet fed lamb was due to the formation of a Cu-Mo complex in the serum, which was unavailable to the animal and thus excreted in the urine.

8. The results of the study indicated that, liver Cu concentrations were the best measure of Cu status. Serum Cu concentration and serum CpOx activity, as a conventional measure of Cu status, were not reliable if Mo and S were present in excess relative to the Cu in the diet. Serum TCAIS-Cu has shown potential as a tool in determining Cu status of sheep, however, further studies must be conducted in order to determine the level of serum TCAIS-Cu which is associated with Cu deficiency.

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A P P E N D I X

APPENDIX I

Analysis of variance tables for parameters measured in ram lambs fed ad libitum pelleted diets either unsupplemented (Control) or supplemented with 10 mg Mo/Kg and 2 g S/Kg alone (0 Cu) or with 10 or 20 mg Cu/Kg (10 Cu, 20 Cu) for 16 weeks.

Table I-1. Analysis of variance for Liver Cu (µg/g W.B.), Body weight (Kg), ADG (g), and Serum Cu (µg/ml) of ram lambs fed pelleted diets for 16 weeks

TRAIT	PLOT	SOURCE	df	TYPE III MS[a]	F	PR>F[b]
Liver Cu	M[c]	D[d]	3	59174.07	20.35	0.0001
		Error A[e]	24	2907.08		
	S[f]	T[g]	4	6969.50	26.59	0.0001
		D X T	12	7704.71	29.40	0.0001
		Error B[h]	94	262.07		
Body weight	M	D	3	15.162	0.06	0.9805
		Error A	24	254.535		
	S	T	4	4502.350	747.22	0.0001
		D X T	12	5.528	0.92	0.5328
		Error B	94	6.025		
ADG	M	D	3	1458.01	0.20	0.8986
		Error A	24	7469.96		
	S	T	3	274823.86	35.59	0.0001
		D X T	9	10034.98	1.30	0.2528
		Error B	69	7721.48		
Serum Cu	M	D	3	0.4742	17.16	0.0001
		Error A	24	0.0276		
	S	T	4	0.1383	11.35	0.0001
		D X T	12	0.0577	4.74	0.0001
		Error B	94	0.0121		

[a]provides the highest level of protection against error; [b]level of significance; [c]main plot; [d]diet; [e]Error A = animal (diet); [f]sub plot; [g]time and [h]Error B = animal (diet X time).

Table I-2. Analysis of variance of CpOx (ΔA/min/ml), Serum TCAS Cu (µg/ml), Serum TCAIS Cu (µg/ml) and Serum Mo (µg/ml) of ram lambs fed pelleted diets for 16 weeks

TRAIT	PLOT	SOURCE	df	TYPE III MS[a]	F	PR>F[b]
CpOx	M[c]	D[d]	3	0.002584	7.65	0.0009
		Error A[e]	24	0.000337		
	S[f]	T[g]	4	0.000745	13.36	0.0001
		D X T	12	0.000256	4.60	0.0001
		Error B[h]	94	0.000512		
Serum TCAS	M	D	3	0.1426	6.89	0.0017
		Error A	24	0.0207		
	S	T	4	0.1003	12.76	0.0001
		D X T	12	0.0249	3.17	0.0008
		Error B	92	0.0078		
Serum TCAIS Cu	M	D	3	0.6745	45.11	0.0001
		Error A	24	0.0149		
	S	T	4	0.1185	17.69	0.0001
		D X T	12	0.0609	9.10	0.0001
		Error B	92	0.0067		
Serum Mo	M	D	3	0.6534	32.90	0.0001
		Error A	24	0.0198		
	S	T	2	0.0550	5.85	0.0059
		D X T	5	0.0239	2.55	0.0431
		Error B	40	0.0094		

[a]provides the highest level of protection against error; [b]level of significance; [c]main plot; [d]diet; [e]Error A = animal (diet); [f]sub plot; [g]time and [h]Error B = animal (diet X time).

APPENDIX II

The quantities and concentration of copper and molybdenum obtained in the diet, feces and urine for copper and molybdenum absorption trial of ram lambs fed *ad libitum* pelleted diets either unsupplemented (Control) or supplemented with 10 mg Mo and 2 g S/kg alone (0 Cu) or with 10 or 20 mg Cu/kg (10 Cu, 20 Cu) during 5 days in each collection period (week 4, 8, 12 and 16).

Table II-1 The quantities and concentration of copper and molybdenum in the diet, feces and urine of ram lambs fed pelleted diets during 5 day collection period in week 4

	Control	0 Cu	10 Cu	20 Cu
Dietary Cu conc. (mg/kg)	10.82	7.14	16.10	23.27
Dietary Mo conc. (mg/kg)	4.27	10.22	7.80	12.24
Feed intake (kg, DM)	8.02	9.85	9.98	10.68
Total Cu intake (mg)	86.77	70.33	160.68	248.52
Total Mo intake (mg)	34.24	100.67	77.84	130.72
Fecal Cu conc. (mg/kg)	32.21	28.31	58.78	85.65
Fecal Mo conc. (mg/kg)	12.35	25.22	23.20	40.11
Fecal output (kg, DM)	2.39	2.57	2.72	2.75
Total fecal Cu output (mg)	76.98	72.75	159.88	235.54
Total fecal Mo output (mg)	29.51	64.81	63.10	110.30
Urinary Cu conc. (μg/ml)	0.073	0.102	0.088	0.074
Urinary Mo conc. (μg/ml)	0.850	3.866	2.074	1.373
Urine output (liter)	5.480	7.650	5.985	5.200
Total urinary Cu output (mg)	0.400	0.780	0.526	0.385
Total urinary Mo output (mg)	4.658	29.57	12.413	7.14
Apparent Cu absorption (%)	11.33	-3.55	0.49	5.21
Apparent Mo absorption (%)	13.81	35.62	18.94	15.61

Table II-2 The quantities and concentration of copper and molybdenum in the diet, feces and urine of ram lambs fed pelleted diets during 5 day collection period in week 8

	Control	0 Cu	10 Cu	20 Cu
Dietary Cu conc. (mg)	8.76	6.30	16.43	24.11
Dietary Mo conc. (mg)	2.08	10.96	16.17	13.42
Feed intake (kg, DM)	8.93	9.48	11.42	10.09
Total Cu intake (mg)	78.23	59.72	187.63	243.27
Total Mo intake (mg)	18.57	103.90	184.66	135.41
Fecal Cu conc. (mg/kg)	21.86	21.22	52.49	74.84
Fecal Mo conc. (mg)	5.27	23.21	42.77	37.46
Fecal output (kg, DM)	3.17	2.91	3.59	3.02
Total fecal Cu output (mg)	69.29	61.75	188.44	226.02
Total fecal Mo output (mg)	16.70	67.54	153.54	113.13
Urinary Cu conc. (μg/ml)	0.093	0.107	0.091	0.097
Urinary Mo conc. (μg/ml)	0.385	4.178	3.353	3.028
Urine output (liter)	5.750	7.530	6.015	5.760
Total urinary Cu output (mg)	0.535	0.806	0.547	0.558
Total urinary Mo output (mg)	2.214	31.46	20.17	17.44
Apparent Cu absorption (%)	11.38	-3.43	-0.39	7.09
Apparent Mo absorption (%)	10.47	35.00	16.82	16.47

Table II-3 The quantities and concentration of copper and molybdenum in the diet, feces and urine of ram lambs fed pelleted diets during 5 day collection period in week 12

	Control	0 Cu	10 Cu	20 Cu
Dietary Cu conc. (mg/kg)	6.88	7.43	17.78	21.32
Dietary Mo conc. (mg/kg)	1.60	12.10	13.31	12.57
Feed intake (kg, DM)	8.65	7.52	8.01	9.70
Total Cu intake (mg)	59.51	55.87	142.42	206.80
Total Mo intake (mg)	13.84	90.99	106.61	121.93
Fecal Cu conc. (mg/kg)	18.32	23.81	46.07	65.57
Fecal Mo conc. (mg/kg)	4.30	24.43	30.18	35.29
Fecal output (kg, DM)	2.76	2.39	3.02	2.91
Total fecal Cu output (mg)	50.56	56.90	139.13	190.81
Total fecal Mo output (mg)	11.87	58.39	91.14	102.69
Urinary Cu conc. (μg/ml)	0.114	0.141	0.115	0.139
Urinary Mo conc. (μg/ml)	0.360	3.023	2.521	2.185
Urine output (liter)	5.200	7.375	6.000	5.490
Total urinary Cu output (mg)	0.593	1.040	0.690	0.763
Total urinary Mo output (mg)	1.872	22.29	15.12	11.99
Apparent Cu absorption (%)	15.04	-1.88	2.32	7.73
Apparent Mo absorption (%)	14.23	35.82	14.50	15.75

Table II-4 The quantities and concentration of copper and molybdenum in the diet, feces and urine of ram lambs fed pelleted diets during 5 day collection period in week 16

	Control	0 Cu	10 Cu	20 Cu
Dietary Cu conc. (mg/kg)	8.28	6.85	14.92	23.93
Dietary Mo conc. (mg/kg)	2.08	12.95	15.33	16.92
Feed intake (kg, DM)	6.96	5.56	8.85	6.42
Total Cu intake (mg)	57.63	38.08	132.04	153.63
Total Mo intake (mg)	14.47	72.00	135.67	108.62
Fecal Cu conc. (mg/kg)	21.07	23.09	44.50	71.76
Fecal Mo conc. (mg/kg)	5.26	25.26	38.75	46.94
Fecal output (kg, DM)	2.36	1.68	2.93	1.99
Total fecal Cu output (mg)	49.72	38.79	130.38	142.80
Total fecal Mo output (mg)	12.41	42.43	113.54	93.41
Urinary Cu conc. (μg/ml)	0.095	0.108	0.095	0.117
Urinary Mo conc. (μg/ml)	0.390	3.547	2.887	3.117
Urine output (liter)	5.490	6.480	5.475	4.295
Total urinary Cu output (mg)	0.521	0.704	0.519	0.502
Total urinary Mo output (mg)	2.141	23.020	15.806	13.387
Apparent Cu absorption (%)	13.72	-1.84	1.28	7.06
Apparent Mo absorption (%)	14.18	40.84	16.29	13.99

APPENDIX III

Computer printout of raw data collected from 28 ram lambs fed ad libitum pelleted diets either unsupplemented (Control) or supplemented with 10 mg Mo/Kg and 2 g S/Kg alone (0 Cu) or with 10 or 20 mg Cu/Kg (10 Cu, 20 Cu) for 16 weeks.

List of abbreviation are coded as:

OBS[1]	observation number
ANML	animal number
DIET	1 = Control 2 = 0 Cu 3 = 10 Cu 4 = 20 Cu
TIME	1 = week 0 2 = week 4 3 = week 8 4 = week 12 5 = week 16
LIVR	liver Cu (µg/g W.B.)
SERM	serum Cu (µg/ml)
CpOx	ceruloplasmin oxidase (ΔA/min/ml serum)
WEGT	body weight (Kg)
ADG	average daily gain (g)
TCAS	serum TCA soluble Cu (µg/ml)
TCAIS	serum TCA insoluble Cu (µg/ml)
MOLY[2]	serum Mo (µg/ml)

[1] observation 65 and 135 are missing as animals # 41 and # 33 died one day after liver biopsy in week 12.

[2] serum for Mo analysis was available only the last three collection periods (week 8,12 and 16).

OBS	ANML	DIET	TIME	LIVR	SERM	CPOX	WEGT	ADG	TCAS	TCAIS	MOLY
1	20	1	1	127.49	0.841	0.0416	49.090	.	0.7505	0.091	.
2	20	1	2	119.89	1.101	0.0722	55.454	265	1.0472	0.054	.
3	20	1	3	128.27	0.925	0.0484	65.454	322	0.7965	0.129	1.187
4	20	1	4	131.15	0.986	0.0566	73.636	292	0.9647	0.022	0.285
5	20	1	5	188.90	1.130	0.0646	79.545	203	0.9424	0.188	0.342
6	25	1	1	86.51	0.845	0.0462	38.181	.	0.7882	0.057	.
7	25	1	2	104.08	0.917	0.0544	44.090	246	0.8608	0.057	.
8	25	1	3	110.70	1.067	0.0630	55.909	381	0.9101	0.157	0.723
9	25	1	4	131.07	0.833	0.0584	67.272	405	0.7478	0.086	0.176
10	25	1	5	188.93	0.858	0.0504	70.454	109	.	.	0.140
11	55	1	1	98.77	0.858	0.0422	35.454	.	0.7599	0.099	.
12	55	1	2	114.83	1.131	0.0724	45.454	416	1.0207	0.111	.
13	55	1	3	140.14	0.916	0.0506	56.818	366	0.7642	0.157	0.568
14	55	1	4	184.02	0.933	0.0682	68.181	405	0.8102	0.123	0.096
15	55	1	5	212.51	1.120	0.0662	75.909	266	0.9901	0.130	0.174
16	49	1	1	83.91	0.845	0.0420	32.727	.	0.7330	0.112	.
17	49	1	2	71.31	0.971	0.0628	32.727	0	0.9076	0.064	.
18	49	1	3	75.63	0.991	0.0526	42.272	308	0.8259	0.166	1.666
19	49	1	4	107.64	1.028	0.0684	47.727	194	0.8530	0.175	0.145
20	49	1	5	168.01	1.072	0.0694	51.818	141	0.8869	0.186	0.206
21	51	1	1	103.73	0.885	0.0448	32.272	.	0.7419	0.143	.
22	51	1	2	101.21	1.193	0.0864	42.727	435	1.1321	0.061	.
23	51	1	3	164.05	1.097	0.0720	46.818	132	0.9395	0.158	1.072
24	51	1	4	211.85	1.064	0.0780	53.636	243	0.8471	0.217	0.088
25	51	1	5	214.63	1.068	0.0760	58.636	172	0.9237	0.145	0.090
26	54	1	1	91.93	0.733	0.0298	29.090	.	0.6751	0.058	.
27	54	1	2	82.31	1.127	0.0620	40.000	454	1.0055	0.122	.
28	54	1	3	137.90	0.791	0.0432	49.090	293	0.6487	0.143	1.555
29	54	1	4	166.01	0.879	0.0586	59.090	357	0.7089	0.171	0.054
30	54	1	5	281.29	0.951	0.0560	61.818	94	0.8450	0.106	0.173
31	50	1	1	82.94	1.047	0.0474	25.454	.	0.9937	0.054	.
32	50	1	2	84.36	0.977	0.0592	35.454	416	0.9134	0.064	.
33	50	1	3	96.04	0.846	0.0492	45.454	322	0.7178	0.129	2.111
34	50	1	4	129.25	0.870	0.0626	55.000	341	0.7703	0.100	0.257
35	50	1	5	212.15	0.917	0.0612	60.454	188	0.7919	0.126	0.198

OBS	ANML	DIET	TIME	LIVR	SERM	CPOX	WEGT	ADG	TCAS	TCAIS	MOLY
36	15	2	1	99.97	0.820	0.0346	47.272	.	0.7817	0.039	.
37	15	2	2	68.43	1.561	0.0396	55.909	359	0.7282	0.833	.
38	15	2	3	48.29	1.141	0.0260	65.000	293	0.6127	0.529	0.754
39	15	2	4	27.85	1.379	0.0216	75.454	373	0.5195	0.860	0.822
40	15	2	5	41.59	1.242	0.0492	75.909	15	0.7213	0.521	0.565
41	26	2	1	142.28	0.803	0.0406	39.090	.	0.7656	0.038	.
42	26	2	2	81.26	1.697	0.0392	46.363	303	0.8699	0.828	.
43	26	2	3	50.43	1.130	0.0296	56.818	337	0.6772	0.453	0.716
44	26	2	4	40.74	1.063	0.0408	65.000	292	0.6225	0.441	0.422
45	26	2	5	43.13	1.340	0.0414	70.909	203	0.6200	0.720	0.748
46	47	2	1	100.16	0.853	0.0400	35.454	.	0.7618	0.092	.
47	47	2	2	52.67	1.236	0.0454	43.636	341	0.7761	0.460	.
48	47	2	3	23.33	1.113	0.0490	53.636	322	0.8303	0.283	0.376
49	47	2	4	22.59	1.014	0.0660	62.727	324	0.7573	0.257	0.317
50	47	2	5	16.89	1.067	0.0620	66.363	125	0.7627	0.305	0.338
51	48	2	1	95.92	0.907	0.0386	34.545	.	0.7710	0.136	.
52	48	2	2	50.63	1.296	0.0330	45.000	435	0.6603	0.636	.
53	48	2	3	25.53	1.268	0.0362	55.454	337	0.7269	0.542	0.607
54	48	2	4	27.16	1.148	0.0302	67.727	438	0.5133	0.635	0.727
55	48	2	5	17.05	1.235	0.0536	72.272	156	0.7062	0.529	0.601
56	10	2	1	128.93	0.993	0.0502	33.636	.	0.8627	0.131	.
57	10	2	2	71.42	1.506	0.0478	41.818	341	1.0771	0.429	.
58	10	2	3	41.11	1.093	0.0218	51.363	308	0.6742	0.419	0.548
59	10	2	4	33.35	1.034	0.0420	57.727	227	0.6728	0.362	0.457
60	10	2	5	32.36	1.200	0.0474	56.818	-31	0.7909	0.410	0.543
61	41	2	1	98.84	0.943	0.0406	30.000	.	0.8054	0.138	.
62	41	2	2	49.03	1.208	0.0316	39.090	378	0.5440	0.664	.
63	41	2	3	36.04	0.928	0.0402	42.272	.	0.6934	0.235	0.497
64	41	2	4	34.60	1.084	0.0314	52.272	357	0.5723	0.512	0.513
65	41	2	5	.	.	.	.	.	.	.	.
66	58	2	1	70.18	0.981	0.0408	22.727	.	0.8429	0.139	.
67	58	2	2	29.06	1.341	0.0456	30.000	303	0.7740	0.567	.
68	58	2	3	13.13	1.238	0.0284	40.909	352	0.6623	0.576	0.723
69	58	2	4	8.97	0.920	0.0510	53.181	438	0.6285	0.292	0.315
70	58	2	5	7.13	0.797	0.0332	58.181	172	0.5695	0.230	0.286

OBS	ANML	DIET	TIME	LIVR	SERM	CPOX	WEGT	ADG	TCAS	TCAIS	MOLY
71	23	3	1	108.31	0.851	0.0440	45.454	.	0.8059	0.046	.
72	23	3	2	51.90	0.726	0.0296	55.909	435	0.6543	0.072	.
73	23	3	3	54.07	0.888	0.0174	61.818	190	0.6484	0.240	0.122
74	23	3	4	48.63	0.903	0.0350	72.727	389	0.5992	0.304	0.248
75	23	3	5	53.27	0.701	0.0250	73.181	15	0.5111	0.190	0.202
76	37	3	1	132.36	0.825	0.0382	40.000	.	0.7146	0.111	.
77	37	3	2	82.28	0.902	0.0460	50.000	416	0.8469	0.056	.
78	37	3	3	69.18	0.801	0.0256	60.909	352	0.6399	0.162	0.202
79	37	3	4	63.39	0.843	0.0456	70.909	357	0.6946	0.149	0.178
80	37	3	5	69.65	0.810	0.0400	72.727	62	0.6747	0.136	0.122
81	39	3	1	75.92	0.878	0.0396	35.454	.	.	.	.
82	39	3	2	44.42	1.160	0.0384	45.454	416	0.8899	0.271	.
83	39	3	3	41.89	0.821	0.0308	52.272	219	0.6524	0.169	0.244
84	39	3	4	38.76	0.946	0.0398	62.272	357	0.6154	0.331	0.299
85	39	3	5	38.51	0.900	0.0440	65.000	94	0.7350	0.165	0.177
86	34	3	1	77.13	0.810	0.0392	34.090	.	0.7871	0.023	.
87	34	3	2	37.48	0.970	0.0244	42.272	341	0.7869	0.184	.
88	34	3	3	28.73	0.777	0.0266	51.363	293	0.6280	0.149	0.103
89	34	3	4	20.99	0.736	0.0328	61.363	357	0.5393	0.197	0.260
90	34	3	5	21.87	0.790	0.0400	65.000	125	0.6375	0.153	0.097
91	43	3	1	99.58	0.977	0.0514	31.818	.	0.8866	0.091	.
92	43	3	2	62.27	1.053	0.0480	38.181	265	0.8564	0.197	.
93	43	3	3	39.22	0.881	0.0386	47.727	308	0.6903	0.191	0.127
94	43	3	4	35.63	0.891	0.0346	52.727	178	0.6706	0.221	0.315
95	43	3	5	59.08	0.995	0.0536	58.181	188	0.7989	0.197	0.156
96	45	3	1	101.14	1.056	0.0426	30.000	.	0.9948	0.062	.
97	45	3	2	72.46	0.968	0.0396	38.181	340	0.7585	0.210	.
98	45	3	3	42.11	0.703	0.0266	48.181	322	0.5385	0.165	0.325
99	45	3	4	54.10	0.778	0.0348	59.090	389	0.5125	0.266	0.292
100	45	3	5	52.67	0.906	0.0276	62.272	109	0.5520	0.354	0.144
101	40	3	1	85.79	1.042	0.0620	27.272	.	0.9361	0.106	.
102	40	3	2	48.79	1.022	0.0548	35.454	341	0.8400	0.182	.
103	40	3	3	48.20	0.840	0.0460	43.181	249	0.6940	0.146	0.166
104	40	3	4	65.82	0.951	0.0718	50.000	243	0.7206	0.231	0.108
105	40	3	5	74.94	1.123	0.0650	55.454	188	0.8781	0.245	0.225

OBS	ANML	DIET	TIME	LIVR	SERM	CPOX	WEGT	ADG	TCAS	TCAIS	MOLY
106	11	4	1	106.18	0.877	0.0490	44.545	.	0.7971	0.080	.
107	11	4	2	99.75	0.771	0.0382	50.454	246	0.7156	0.056	.
108	11	4	3	98.86	0.856	0.0376	60.000	308	0.7625	0.094	0.155
109	11	4	4	107.15	0.955	0.0540	70.000	357	0.7200	0.235	0.107
110	11	4	5	112.24	0.933	0.0546	71.363	47	0.7669	0.167	0.067
111	19	4	1	140.63	0.875	0.0512	44.545	.	0.7867	0.089	.
112	19	4	2	152.80	0.892	0.0482	53.636	378	0.8348	0.058	.
113	19	4	3	151.84	0.992	0.0586	64.545	351	0.7930	0.199	0.105
114	19	4	4	208.97	0.940	0.0554	70.454	211	0.8244	0.116	0.062
115	19	4	5	224.72	1.212	0.0534	75.454	172	0.9102	0.302	0.000
116	32	4	1	94.02	0.908	0.0394	32.272	.	0.7842	0.124	.
117	32	4	2	61.14	0.938	0.0432	43.181	454	0.6580	0.280	.
118	32	4	3	74.14	0.760	0.0290	51.363	264	0.7074	0.053	0.613
119	32	4	4	101.96	0.653	0.0378	61.363	357	0.4963	0.157	0.205
120	32	4	5	138.89	0.813	0.0446	62.272	31	0.7145	0.099	0.000
121	42	4	1	89.72	0.842	0.0428	31.818	.	0.7281	0.114	.
122	42	4	2	60.29	0.891	0.0418	34.545	113	0.8120	0.079	.
123	42	4	3	64.07	0.766	0.0328	55.909	689	0.7614	0.005	0.276
124	42	4	4	78.40	0.891	0.0610	63.181	259	0.7658	0.126	0.113
125	42	4	5	99.96	0.681	0.0428	68.636	188	0.5830	0.098	0.021
126	56	4	1	88.43	0.807	0.0294	30.454	.	0.7094	0.098	.
127	56	4	2	37.12	0.811	0.0368	40.000	397	0.7355	0.076	.
128	56	4	3	56.76	0.633	0.0184	52.727	410	0.4889	0.145	0.196
129	56	4	4	104.41	0.708	0.0262	63.181	373	0.5820	0.126	0.136
130	56	4	5	112.01	0.861	0.0220	67.727	156	0.5285	0.333	0.018
131	33	4	1	138.69	1.013	0.0434	30.000	.	0.9418	0.072	.
132	33	4	2	107.31	0.827	0.0344	37.727	322	0.7665	0.061	.
133	33	4	3	78.21	0.670	0.0224	49.545	381	0.5588	0.112	0.161
134	33	4	4	99.31	0.861	0.0554	55.000	194	0.7632	0.098	0.162
135	33	4	5	.	.	.	.	.	.	.	.
136	44	4	1	129.90	0.973	0.0522	29.090	.	0.8260	0.147	.
137	44	4	2	113.70	0.900	0.0392	37.272	341	0.8111	0.089	.
138	44	4	3	134.10	0.795	0.0338	50.000	410	0.6873	0.108	0.188
139	44	4	4	192.23	1.238	0.0828	60.909	389	1.0550	0.183	0.110
140	44	4	5	227.88	1.023	0.0624	64.090	109	0.8462	0.177	0.039