EXPERIMENTAL CHRONIC COPPER POISONING IN SHEEP

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by

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as cause changes in chemical composition of the membrane. The latter may be the cause of intravascular hemolysis observed in copper poisoning (Lubin and Desforges, 1972; Soli and Nafstad, 1976). Soli and Nafstad (1976) observed membrane interruptions in erythrocytes containing Heinz bodies in electron microscopic sections. This evidence was used to support the observed intravascular hemolysis.

The present scanning electron work demonstrated that formation of Heinz bodies immediately before hemolysis was accompanied by marked distortion of erythrocyte membranes with occasional spiculated erythrocytes. Several erythrocytes had membrane interruptions which presented as elevated patches on the surface of erythrocyte membranes. Some cells were rounded which can be taken as evidence of fragmentation (Bessis et al., 1973). Hemolytic crisis was accompanied by increased spherocytosis. Membrane fragmentation was more marked with membrane breaks spread over the entire surface of the erythrocyte.

On the basis of the results from scanning electron microscopy, it would be difficult to speculate on the actual cause of membrane interruptions observed. It is conceivable that membrane injury by peroxides and Heinz body attachment could cause these interruptions which in turn lead to the observed intravascular hemolysis. Non-specific attachment of proteins to erythrocyte membranes caused by copper ions could result in a Coombs positive reaction as observed by Jandl and Simmons (1957), but this would only result in increased sequestration of erythrocytes in the RES.

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Copper poisoning was produced in sheep by feeding them copper sulfate ($CuSO_4 \cdot 5H_2O$). In sheep given daily oral doses of 1.5 g $CuSO_4 \cdot 5H_2O$ as 1 percent solution, severe signs of hemolysis appeared in 24 to 26 days. Signs of hemoglobinuria, hemoglobinemia and jaundice were accompanied by a rapid drop in hematocrit to a low of 6 percent in four days. This disease was subsequently called subacute copper poisoning.

Sheep fed copper sulfate as a powder added to the ration hemolyzed after day 42. The hemolytic crises were mild and some sheep went through two crises before they were killed. One sheep was fed copper for 131 days without signs of a crisis. This regimen was referred to as chronic copper poisoning.

Aspects of chronic copper poisoning studied included plasma assays for liver and kidney function, total plasma copper concentration and ceruloplasmin activity. Histological changes in the liver and kidney were examined at postmortem. Erythrocyte (RBC) pentose phosphate shunt pathway enzymes and other hematologic parameters were recorded. RBC

morphology was studied using scanning electron microscopy.

Subcellular liver copper storage was determined by differential centrifugation of post-mortem liver homogenate and examination of glutaraldehyde fixed liver samples using transmission electron microscopy.

Increased activities of plasma glutamate oxaloacetate transaminase (GOT), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH) and sulfobromophthalein (BSP) clearance time during the hemolytic crisis implied destruction of liver tissue which was confirmed by histology. Increase in liver function tests prior to hemolytic crisis was noted in the sub-acutely poisoned sheep and in one of the five sheep that were chronically poisoned. A similar trend was noted in plasma copper concentration. Plasma ceruloplasmin activity did not change. Liver function tests and plasma copper for early diagnosis of chronic copper poisoning may be of use in animals which would later undergo severe crises.

Increases in plasma blood urea nitrogen (BUN) and creatinine have a prognostic importance in hemolytic crisis.

Erythrocyte glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) remained unaffected until after the hemolytic crisis when their activities increased due to an increase in circulating immature erythrocytes. Erythrocyte reduced glutathione (GSH) decreased by nearly 50 percent during crisis. Decrease of GSH was not attributable to *in vivo* inhibition of pentose phosphate shunt enzymes.

Scanning electron microscopy revealed that erythrocytes retained a normal biconcave shape up to 48 hours before hemolytic crisis. Changes observed on the day preceeding the crisis included irregularly distributed swellings attributed to Heinz bodies, fragmentation of the erythrocyte membrane and spicule formation in a few cells. Hemolytic crisis was associated with marked distortion and rounding due to increased fragmentation. Many cells contained pits where Heinz bodies had been located previously.

Sheep with normal liver copper concentrations stored nearly 40 percent in the 14,000 g pellet. In chronically poisoned sheep, over 40 percent of liver copper appeared in the nuclear fraction (600 g pellet), while subacutely poisoned sheep accumulated copper mostly in the cytosol.

Liver cytosol of sheep with normal or slightly elevated copper concentration had most copper in a high molecular weight (MW) fraction (75,000) while a low molecular weight fraction (12,000) showed increased binding in copper poisoned sheep. A fourth copper fraction (MW approximately 5000) was recorded in copper poisoned sheep. This fraction bound the most copper in subacutely poisoned sheep and may be related to appearance and severity of hemolytic crisis signs.

D-penicillamine, 0.5 g as a 2 percent solution given daily as an intravenous (IV) injection for four days, did not significantly lower hepatic copper content in housed Southdown sheep. A mean of 3.8 mg copper was mobilized through the urine in four days. The IV treatment did not affect the

health of the sheep.

To my grandmother, Mary Thogori Mathenge.

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CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

General Introduction

Copper is essential to life in mammals, plants and lower forms of organisms. It has varied and numerous biologic effects both as an essential element and as a toxicant. The effects are greatly influenced by the form and level of exposure, species of organism, diet, disease state and individuality.

When eaten in large doses, copper causes gastrointestinal irritation in all mammals. Collapse and death follow in 24 to 48 hours.

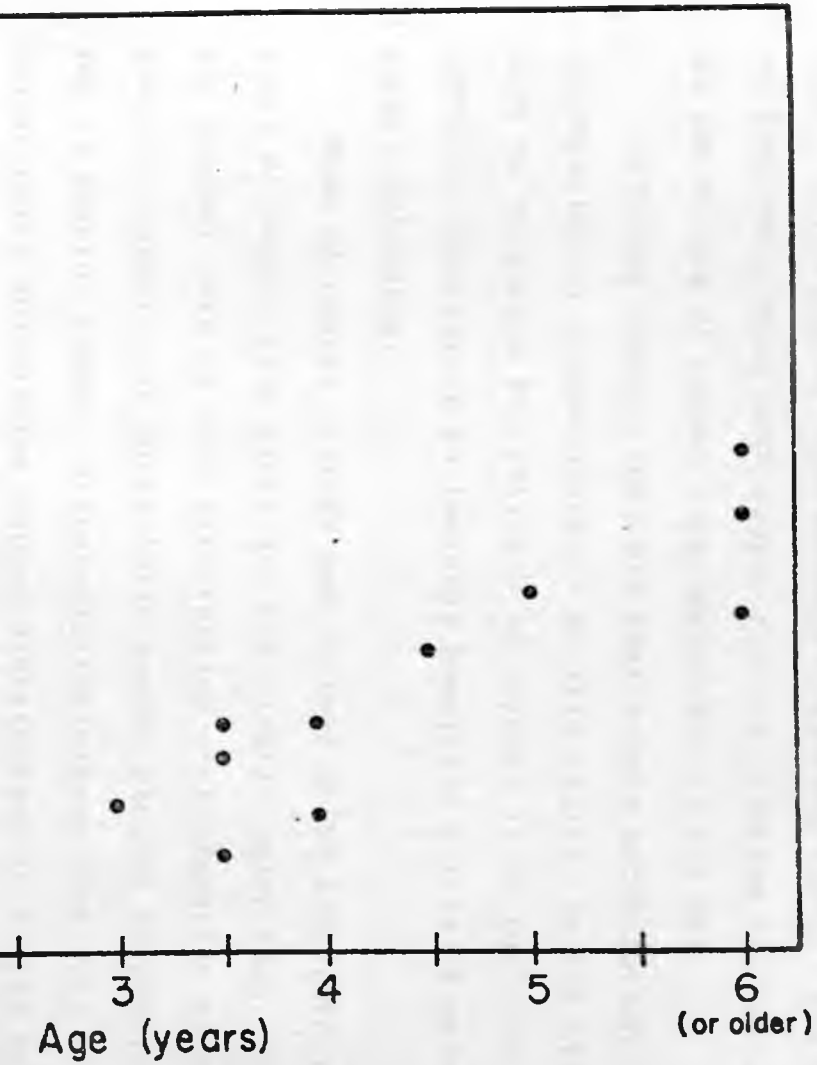
Chronic copper poisoning is characterized by a long period of hepatic copper accumulation followed by an acute intravascular hemolysis associated with sudden increase in plasma copper concentration. Affected animals may die within 24 to 48 hours after signs are seen or may survive several crises before they succumb.

Chronic copper poisoning is essentially a problem of sheep under practical farm conditions. Sheep accumulate copper in their livers when fed copper slightly in excess of requirements. Sheep of all ages are affected but a higher incidence is reported in housed than in pastured sheep. A survey done on housed Southdown sheep showed that older sheep accumulated more copper than lambs (Fig. 1-1).

In the present work we studied several aspects of chronic

Figure 1-1. Correlation between liver copper concentration and age of housed Southdown sheep (r = 0.74).

Liver Copper (µg/gm DM)
2000
1500
1000
500
1
2


3
4
5
6
(or older)
Age (years)

copper poisoning in experimentally poisoned sheep. The effect of drenching sheep with copper sulfate solution was compared to the effect of copper supplementation in the diet.

Previous reports indicate that copper accumulation is accompanied by degeneration of hepatic cells. In the present work we evaluated the efficacy of several liver function tests in forecasting an imminent hemolytic crisis of chronic copper poisoning.

Mode of copper storage and factors which instigate release of copper from liver are not clearly understood. In the present work we used differential centrifugation techniques to characterize subcellular copper storage in symptomatic and in control sheep. Histologic techniques were used to characterize subcellular copper distribution in copper toxicity.

Pathogenesis of the hemolytic anemia in chronic copper poisoning was studied by analysis of glutathione dependent pentose phosphate shunt enzymes. Erythrocyte morphology was studied with the scanning electron microscope.

Literature Review

Copper Metabolism in Sheep

Sites for copper absorption in ruminants have not been recorded. In other species, the small intestine is the most important absorption site. Absorption occurs equally in the intestine and stomach of rats while some copper absorption has been observed in the colon of the pig (Underwood, 1977).

Copper availability in ruminants is very variable and is mostly determined by age of the animal and level of certain other dietary factors. Absorption of copper is highest in the newborn lamb (71%) and drops gradually to 47 percent at weaning. Thereafter, digestibility is rapidly reduced to adult levels of less than 10 percent (Suttle, 1973). Copper availability is between 4 and 10 percent in adult sheep (Smith et al., 1968; Dick, 1954).

Mechanism of copper absorption is incompletely understood. Crampton et al. (1965) observed that there were two of copper absorption. A small proportion of copper was transported by an energy dependent process. This involved rapid transfer of copper which peaked to a constant level and was maintained over several hours (Marceau et al., 1970). The energy dependent process was later shown to be mediated by L-amino acids whose absorption is also energy dependent (Kirchgessner and Grassmann, 1970).

The other fraction of copper is bound to intestinal mucosa and subsequently absorbed into the plasma. This component is thought to be mediated by metallothionein, a sulfhydryl rich protein with molecular weight (MW) of 10,000 (Evans, 1973). Van Campen and Scaife (1967) demonstrated that zinc interfered with copper absorption in the duodenum. Starcher (1969) identified in the chick duodenum a protein with molecular weight of 10,000 which bound cadmium, zinc and copper. Evans and Cornatzer (1970) found that orally administered ^{64}Cu and

^{65}Zn were associated with the same protein in the duodenum of the rat. They isolated two proteins which were also isolated from bovine duodenum and liver (Evans et al., 1970a). One fraction of MW 35,000 had the properties of superoxide dismutase (McCord and Fridovich, 1969). The other copper binding protein was similar to the metallothionein isolated from equine kidney (Kagi and Vallee, 1960, 1961) and human kidney (Pulido et al., 1966). Highly purified metallothionein contains 26 sulfhydryl groups and binds metals by formation of mercaptides.

Intestinal metallothionein may have at least two functions (Evans, 1973). It may play a passive role by providing binding sites within the intestinal mucosa to insure that adequate supply of the metal is removed from the diet and temporarily stored for subsequent absorption. Secondly, it may protect against absorption of toxic levels of copper.

A mechanism for copper absorption has been proposed by Evans (1973) (Fig. 1-2). As food passes through the gastrointestinal tract, copper dissociates from the macromolecular (MM) species and is released as either ionic copper (Cu^{2+}) or a copper amino acid complex (Cu-AA). A fraction of the ionic copper combines with the available amino acids in the intestinal lumen, after which the bound copper is actively transported across the intestinal mucosa. The portion of copper that transverses the mucosal membrane as an uncomplexed ion combines with the metallothionein, forming a mercaptide

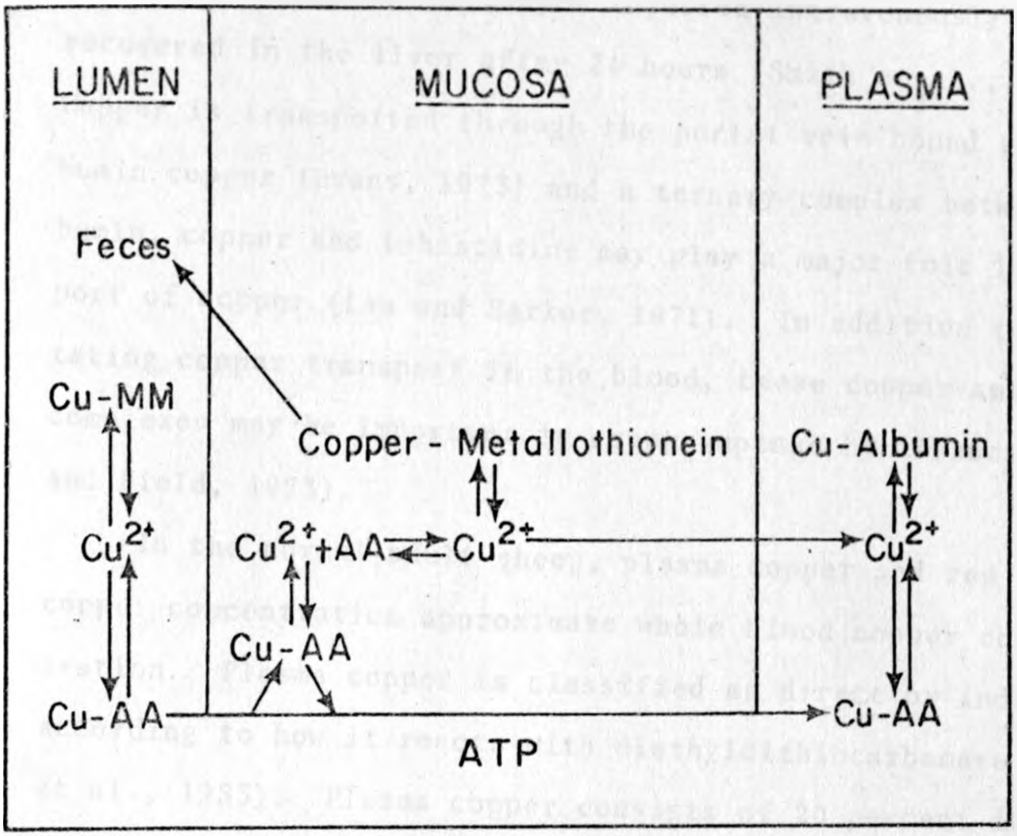


Figure 1-2. Intestinal copper absorption.

with the available sulfhydryl groups. As copper dissociates from the intestinal metallothionein, the metal diffuses directly into the plasma or becomes complexed for subsequent transport to the serosal side. Copper that remains bound to metallothionein is eventually returned to the intestinal lumen concomitant with the migration and shedding of the epithelial cells. This fraction of copper is probably unavailable for reabsorption and is thus excreted.

Absorbed copper is rapidly removed from the blood of sheep as ingestion of 1 g copper sulfate in solution does not elevate blood copper levels (Eden and Green, 1939). Eighty

percent of radioactive copper injected intravenously (IV) is recovered in the liver after 24 hours (Smith et al., 1968). Copper is transported through the portal vein bound to albumin copper (Evans, 1973) and a ternary complex between albumin, copper and L-histidine may play a major role in transport of copper (Lau and Sarkar, 1971). In addition to facilitating copper transport in the blood, these copper-amino acid complexes may be important in copper uptake by tissues (Smith and Field, 1973).

In the normal adult sheep, plasma copper and red cell copper concentration approximate whole blood copper concentration. Plasma copper is classified as direct or indirect according to how it reacts with diethyldithiocarbamate (Gubler et al., 1953). Plasma copper consists of 20 percent direct reacting copper and 80 percent indirect reacting copper. The indirectly reacting copper is in ceruloplasmin and is not exchangeable with ionic copper (Scheinberg and Morrell, 1959). Underwood (1977) observed that the amount of ceruloplasmin copper exchanged daily is too small to account for the copper absorbed from intestine. Transport of copper through the portal vein is, therefore, possible only by binding to albumin and amino acids.

Fetal and newborn animals have lower whole blood copper due to a comparative absence of indirect reacting copper (McCosker, 1968). Normal blood and plasma copper levels vary between individual sheep, breed and hemoglobin types (Wiener

et al., 1976). Normal blood copper levels range from 0.5 to 1.5 ug/ml with most values between 0.8 and 1.20 ug/ml (Underwood, 1977).

Hepatic Copper Metabolism

Copper taken up by the liver is processed in three ways: 1) Preparation for excretion into bile, 2) ceruloplasmin synthesis and 3) storage (Evans, 1973).

The liver is the most important organ in copper homeostasis and it stores 75 percent of all the body copper (Underwood, 1977). In sheep, 80 percent of an injected dose of ^{64}Cu is found in the liver in 24 hours (Smith et al., 1968). Normal liver copper concentration is 100-400 ug/g dry matter (DM). The mean copper concentration varies among breeds (Wiener et al., 1976). Several other factors affect copper storage and they will be discussed as they generally affect copper metabolism.

Bile is the main route for copper excretion. In dogs and pigs 7 to 10 percent of ^{64}Cu is excreted in bile, 0.6 percent in the urine and 1.5 percent is excreted through the intestinal wall (Mahoney et al., 1955). No comparable work has been done in ruminants. Smith et al. (1968) recorded 5 percent excretion in the feces of an intravenous ^{64}Cu dose. This approximates what would be expected even if the portion that comes from the intestinal wall is considered. According to Smith et al. (1968), urinary excretion is normally small (0.54%) and becomes important only in conditions which cause

hypercupruria (Evans, 1973).

Ceruloplasmin synthesis and release supply another route for elimination of copper from the liver. Both plasma ceruloplasmin and hepatic copper are increased by copper injection into rats (Evans et al., 1970b; Evans et al., 1969) and by high copper diet in rabbits (Bagallah et al., 1965). De novo synthesis of ceruloplasmin in response to copper administration has been demonstrated both in the intact adult rat (Evans et al., 1970b) and in isolated rat liver (Owen and Hazelrig, 1966). Increase in dietary copper intake does not seem to increase ceruloplasmin output in the rat (Milne and Weswig, 1968). Apoceruloplasmin is released at the same rate in copper deficient as holoceruloplasmin in the normocupremic rats (Holtzman et al., 1966; Holtzman and Gaumnitz, 1970) but absence of copper increases the turnover of the former resulting in lower concentration.

In sheep, ceruloplasmin levels are poorly correlated with copper states when intake is sufficient, but ceruloplasmin levels are low in copper deficient states (Underwood, 1977). This shows that with excess copper, homeostasis through ceruloplasmin is limited in sheep.

Hepatic Copper Storage

Liver accounts for 75 percent of total body copper in sheep (Dick, 1954). Copper concentration in the liver varies between breeds and blood types (Wiener et al., 1976); normal concentration is 100 to 400 ppm on a dry matter basis

(Underwood, 1977).

Distribution of copper among the subcellular liver organelles varies with age, copper status and the level of several dietary factors. The newborn of most species store most copper in the crude mitochondrial fraction (Porter et al., 1961; Gregoriadis and Sourkes, 1967), while in the adult animal 50 percent of hepatic copper is in the supernatant fraction. Philip (1973) recorded over 50 percent storage of copper in mitochondria and lysosomes of adult sheep with normal copper levels.

Increased copper storage due to feeding high dietary copper (Philip, 1973) or as a result of biliary obstruction results in a redistribution of copper among the subcellular organelles. Increased storage is observed in crude mitochondria and nuclei of the rat liver (Gregoriadis and Sourkes, 1967). Cytosol and microsomes accumulate much less. Similar results were recorded by Philip (1973) in copper loaded sheep. Initially mitochondria accumulated more copper than the nuclei, but, at high copper concentration, the nuclear fraction continued to accumulate copper while mitochondrial accumulation tended to plateau. Significant storage in the cytosol was noticed only at the highest hepatic copper concentrations attained in her experiment. Similar patterns of copper accumulation have been recorded in the rat (Lal and Sourkes, 1971). It is possible that accumulation in the crude mitochondrial fraction is due to accumulation of copper

in the lysosomes as shown by histologic studies of Barka et al., (1964) and Scheuer et al., (1967).

Bremner and Marshall (1974a,b) showed that copper and zinc occur together in the soluble fraction in three main protein fractions with approximate molecular weights (MW) of 75,000, 35,000 and 12,000, designated as fractions I, II and III respectively. At normal hepatic copper concentration, most copper occurred in fraction I (72%) but in heavily loaded livers, there was an increase in the proportion of copper in fraction III. Philip (1973) also noted the three fractions and observed another fraction with MW <10,000 in livers with high hepatic copper. Fraction I, which is uncharacterized, could serve a storage function as 70 percent of the soluble copper in normal liver concentration was in this fraction.

Fraction II is characterized as superoxide dismutase based on its properties which were characteristic of erythrocyprein (McCord and Fridovich, 1969). Fraction III is similar to metallothionein (Kagi and Vallee, 1960, 1961; Evans et al., 1970a). The results of Bremner and Marshall (1974a,b) suggest that accumulation of copper in fraction III is dependent on adequate dietary intake of zinc. Zinc deficiency results in disappearance of copper from this fraction and accumulation in fraction I. There is a strong correlation between total hepatic zinc and total zinc and copper in fraction III. This seems to indicate that zinc is necessary for synthesis of this copper-binding protein In the rat, copper injections

induce synthesis of a copper-chelatin which is different from metallothionein (Winge et al., 1975; and Premakumar et al., 1975a). Copperthioneins have been isolated from pig liver cytosol and have been shown to contain a variable amount of zinc (Bremner and Young, 1976) which could have a stabilizing effect.

Copper-Molybdenum Antagonism

The biologic antagonism between copper and molybdenum was discovered when cattle grazing pastures high in molybdenum developed diarrhea, growth retardation, anemia and achromotrichia, which was prevented by administering copper (Ferguson et al., 1938). Dietary sulfur aggravates this antagonism in ruminants and results in reduced tissue copper. In nonruminants sulfur alleviates the antagonism and increases copper storage (Dick, 1956; Miller and Engel, 1960).

The gastrointestinal tract, especially the rumen, plays a vital role in copper-molybdenum-sulfur antagonism in ruminants (Suttle, 1974c). The effect of dietary molybdenum on copper status is considerably reduced when the rumen is bypassed by giving copper parenterally (Clawson et al., 1972; Suttle and Field, 1974; Suttle, 1974b) or when molybdenum is given by abomasal infusion (Suttle, 1974c).

Interaction of molybdenum, copper and sulfur was demonstrated by Suttle (1974b). Molybdenum alone increased plasma molybdenum but had no effect on the availability of copper. Sulfur alone slightly reduced copper availability. Together,

the two elements reduced copper availability by 50 percent with no elevation of plasma molybdenum. Suttle (1974b) thought that this interaction represented the formation of the copper thiomolybdate complex described by Tridot and Bernard (1962).

Theory by Dowdy and Matrone (1968a) involved formation of a copper-molybdenum (Cu-Mo) complex which was unavailable to the pig. When given intravenously to sheep, it was excreted so that neither of the metals was available (Dowdy and Matrone, 1968b). Though Mills and Mitchell (1971) reported accumulation of copper and molybdenum in tissues in the ratio found in the Cu-Mo complex, when supplemented with excess molybdenum, sheep given the preformed complex did not accumulate the metals proportionately (Dowdy and Matrone, 1968b). The complex is unstable below pH 3, the acidic condition found in the stomach and abomasum (Dowdy et al., 1969).

Molybdenum supplementation increases the direct-reacting fraction of plasma copper and reduces ceruloplasmin levels. (Suttle and Field, 1968; Marcilese et al., 1969). This has been shown to be a true systemic effect of molybdenum in sheep given subcutaneous copper (Suttle and Field, 1974) and is dependent on the level of molybdenum in the diet. This may reflect an impaired copper uptake of absorbed copper by the tissues or it may represent a direct effect of molybdenum on the capacity of plasma proteins to bind copper. Smith and Wright (1973) described a copper complex which was stable in acid pH and its copper could exchange with ^{64}Cu.

Molybdenum supplementation increases copper deposition in the kidneys and increases urinary copper excretion (Suttle, 1974; Suttle and Field, 1968; Marcilese et al., 1969). Copper accumulation in kidneys is thought to result from deposition of the copper-molybdenum complex.

Copper and Zinc Interactions

Trace elements which have similar chemical and physical properties would be expected to show some biological antagonism (Hill and Matrone, 1970). Increased intake of zinc and iron largely eliminated the symptoms of toxicity in pigs on diets high in copper as a growth stimulant (Suttle and Mills, 1966). Pigs maintained on high copper may suffer parakeratotic lesions due to zinc deficiency (Ritchie et al., 1963). Mills (1974) has shown that feeding high levels of zinc (100 ug Zn/g) reduced the risk of copper toxicity when lambs were fed 60 ug Cu and then stressed.

The mechanism of protection is not known but one possible explanation is competition for common sites of absorption. It has been shown that high doses of zinc impair intestinal uptake and transfer of copper (Starcher, 1969; Van Campen and Scaife, 1967). Whanger and Weswig (1970) demonstrated that cadmium, silver and zinc antagonize copper metabolism within hepatic cells. They bypassed intestinal competition by injecting copper into rats. Ceruloplasmin synthesis was inhibited in rats supplemented with the above trace elements.

Accumulation of zinc in livers of rats was accompanied

by a lower concentration of copper in microsomes and the soluble fraction (Whanger and Weswig, 1970). Since ceruloplasmin synthesis occurs in the microsomes, it is conceivable that displacement of copper from this fraction will result in lowered ceruloplasmin synthesis (Whanger and Weswig, 1970). Conversely, copper accumulation in the liver results in redistribution of zinc (Philip, 1973). Copper accumulation in liver lowered zinc levels in the soluble fraction and increased zinc concentration in the nuclear fraction.

The site of zinc-copper antagonism has been identified as metallothionein which is present in the duodenum (Starcher, 1969; Evans et al., 1970a), liver (Evans et al., 1970a; Bremner and Marshall, 1974a,b) and kidneys (Kagi and Vallee, 1961). These elements compete for sulfhydryl binding sites on the metallothionein (Evans et al., 1970a; Bremner and Marshall, 1974b). Increased copper concentration in the liver of ruminants resulted in displacement of zinc from the metallothionein fraction (fraction III) to fraction I (Bremner and Marshall, 1974a,b). Similar displacement of copper occurred when zinc concentration was high.

Effect of Protein Intake

MacPherson and Hemingway (1965) protected sheep from chronic copper poisoning by increasing crude protein intake from 10 percent to 20 percent. Todd (1969) argues that high protein as such may not be the only factor in decreasing copper storage. The supplementary protein, soy beans,

contained high molybdenum levels which could affect metabolism and retention of copper.

Effect of Calcium

A marked depression in copper absorption in sheep can be brought about by high dietary intakes of calcium carbonate (Dick, 1954). The effect of calcium on copper utilization may be attributed to a reduction in absorption capacity due to precipitation of copper as hydroxide. Calcium compounds can raise the pH of the rumen and cause precipitation (Weser and Kirchgessner, 1965).

The Chemical Form of Copper

Lassiter and Bell (1960) studied the availability of orally and intravenously administered copper sulfate, copper nitrate, copper chloride, copper carbonate, cupric oxide and cuprous oxide, all labelled with ^{64}Cu. Copper in copper wire was largely unavailable. The copper in the oxides was less available than that in the water soluble forms or the carbonate.

Changes in the chemical form in which copper exists in foods may affect copper availability. Fresh herbage is significantly less effective in promoting body copper storage than hay or dried herbage of equivalent total copper content (Hartmans and Bosman, 1970). The importance of organic complexes to copper absorption is further demonstrated by the fact that phytate can reduce the assimilation of this element (Davis et al., 1962).

Chronic Copper Poisoning

Occurrence. Toxicity and/or deficiency of copper is complicated by its complex interaction with molybdenum and sulfate. In most cases it is impossible to delineate the toxicity of one and deficiency of the other.

Copper compounds for treating diseases of plants and animals can be a source of copper toxicity. Deaths were reported after sheep ate orchard herbage which had been treated with sprays containing copper (Fincham, 1945; Ogilvie, 1954). Muth (1952) reported mortality in sheep passed through a copper footbath. Calves died after licking walls treated with copper sulfate solution to kill ringworm spores (O'Moore, 1956). Treatment of ewes to prevent swayback in lambs has resulted in copper toxicity (Ishmael et al., 1969; Wiener and Macleod, 1970). These preparations can also be toxic when given to lambs to control delayed swayback (Allcroft et al., 1965). Such deaths may have occurred due to use of a standard amount in animals regardless of body size.

In Australia, chronic copper toxicity has been described in sheep on pasture. The conditions were classified according to their primary effect on sheep (Bull et al., 1956). Phytogenic chronic copper poisoning occurred when sheep consumed forages with an imbalance of copper and molybdenum. This type of poisoning was related to lush growth of Trifolium subterraneum in early autumn. These plants contained little or no molybdenum and sheep grazing on them stored high levels

of copper in the liver which predisposed them to the hemolytic crisis of chronic copper poisoning.

Chronic hepatogenous poisoning in sheep occurs after consumption of plants such as Heliotopium echium and Senecio species which contain pyrrolizidine alkaloids. The alkaloids cause hepatic necrosis with resulting inability to metabolize and excrete normal dietary levels of copper (Underwood, 1977; St. George-Grambauer and Rac, 1962).

Probably related to phytogenous chronic copper toxicity is the situation described by Bull et al., (1956) where consumption of the plant Chondrilla juncea results in chronic copper toxicity. The copper content of the plant varies under different soil and climatic conditions and sometimes is toxic. Fertilizing copper deficient soils with copper compounds in Western Australia causes copper accumulation in Lupinus species which, when consumed by sheep, results in high hepatic copper accumulation and predisposition to lupinosis (Gardiner, 1966; 1967). In South Africa, geeldikkop is accompanied by copper accumulation in the liver, and the hemolytic crisis resembles that of chronic copper poisoning in sheep (Brown, 1968).

Increased incidence of chronic copper poisoning in sheep under confinement has been reported (Todd, 1962; Adamson and Valks, 1969; Todd, 1969). Bracewell (1958) found that the typical hemolytic syndrome in housed sheep receiving a small copper supplement with no molybdenum sulfate continued to occur up to 11 months after withdrawing the copper supplement. Todd (1972) reported the occurrence of copper poisoning in

housed sheep fed oats and barley with low normal levels of copper, 3.9 and 4.9 ppm respectively, but with extremely low levels of molybdenum, less than 0.025 ppm. Consumption of complete feeds with copper-molybdenum imbalance is a man-made problem in the U.S.A. and elsewhere. Molybdenum is not recognized as safe and necessary and, by law, cannot be incorporated into animal feeds (Buck, 1970). Molybdenum is accumulated in tissues and eliminated in milk when given in excessive amounts in the absence of copper and inorganic sulfate; this may create public health hazard. A ration containing supplemental copper but no molybdenum causes copper to accumulate in sheep liver in concentrations of 2000 ppm or greater which could also be hazardous (Adamson and Valks, 1969).

The inclusion in the diets of growing and fattening pigs of large supplements of copper as a growth stimulant has led to discussion of the toxicity of copper to pigs (Wallace et al., 1960) and encouraged detailed studies of factors that influence this (Suttle and Mills, 1966). A further problem is envisioned from the continuous application of pig manure or slurry of high copper content, around 675 mg Cu per kg dry matter (Berryman, 1970), to pastures; the copper content of the herbage may reach toxic levels particularly for young sheep. The form in which most copper is present in the slurry is unknown, but recent observations show it to be of similar availability, when fed to sheep, as that of copper sulfate

(Dalgarno and Mills, 1975). This ready availability is supported by a case of copper poisoning in sheep in Holland that was attributed to hay containing 42 mg Cu per kg dry matter that was made from slurry-treated grassland (Feenstra and Ulsen, 1973). Slight liver damage occurred in ewes and lambs grazing copper treated pastures (Kneale and Howell, 1974), and an evidence of mild toxicity was recorded in cattle given dried grass contaminated with high copper slurry (Blaxter, 1973). However, the evidence against the reality of this proposed hazard seems strong: heavy treatment with slurry increased the copper content of herbage to only about 20 mg per kg dry matter (Batey et al., 1972), and there was no ill-effect on ewes that grazed for three seasons on herbage that was repeatedly contaminated with high copper slurry (Woodside, 1973).

Copper is also used as a growth stimulant (Smith, 1969; Fisher et al., 1973; Lloyd, 1974), as a control for fungal growth in the diet and to reduce aortic rupture in turkeys (Guenther and Carlson, 1974). Poultry waste also poses another source of chronic copper toxicity to sheep. Copper extract from herbage topped with poultry waste was more available than the extract from herbage dressed with pig waste (Suttle and Price, 1976). The copper levels in the herbage after treatment with pig waste were below toxicity level, but after application of poultry waste, the level of available copper was a toxicity hazard.

Susceptibility to chronic copper poisoning. Continued

ingestion of excess copper leads to accumulation of copper in the liver of all animals. Capacity for hepatic storage varies greatly among species and the copper levels that can be tolerated without signs of copper toxicity also vary greatly among species. In sheep and cattle, existence of liver copper levels above very high critical levels is usually followed by a catastrophic liberation of copper into the blood stream with resulting hemolysis and jaundice (Underwood, 1977).

Sheep are most susceptible and can be affected at any age. Most reported cases are in mature animals (Todd, 1969). Cattle seem to become less susceptible to chronic copper poisoning as they mature. Young calves are almost as susceptible as sheep, and outbreaks due to excessive copper intakes have been reported (O'Moore, 1956; Shand and Lewis, 1957). Five grams of copper sulfate given daily to a cow for nine months failed to produce toxicity (Cunningham, 1946). Steers given 8 g copper sulfate in gelatin capsules for 12 months and 12 g for a further four months did not suffer any toxicity (Chapman et al., 1962). Administering the 12 g in solution killed two out of three cows.

Monogastric species are more tolerant to high copper doses. Levels up to 250 mg copper per kg diet are used as growth stimulants for growing and fattening pigs (Suttle and Mills, 1966). When supplemented with high amounts of zinc and iron, levels as high as 750 mg copper per kg diet are tolerated very well (Underwood, 1977).

Chronic copper toxicity is related to excess storage of

copper in the liver. Sheep have accumulated copper even when dietary levels were normal. There is a linear relationship between hepatic copper storage and dietary copper between 4 and 18 mg/kg of feed (Dick, 1954). The pig's dietary threshold for copper accumulation is 70 to 130 ppm (Lucas and Calder, 1957) while that of Brown rats is about 200 ppm (Milne and Weswig, 1968).

Beck (1963) observed that accumulation of copper was more likely due to a slow release of copper from the liver cells of sheep. Milne and Weswig (1968) attributed accumulation of copper in the liver to overloading of excretory mechanisms.

Lal and Sourkes (1971) observed that hepatic copper accumulation was dependent on the dose and duration of administration and not due to overloading of excretory pathways. No work has been done to prove or disprove either theory. Both factors may be important in the case of sheep.

Pathogenesis of chronic copper poisoning. The sheep liver stores copper more readily than that of other species of animals and a copper concentration of 100 to 400 ppm on a dry matter basis is normal (Underwood, 1977). When copper concentration reaches 400 ppm or more, the animal is predisposed to the characteristic hemolytic crisis of copper poisoning. Poisoning is brought about by a sudden release of stored liver copper into the blood. The sudden release of copper by the liver may be spontaneous or may be associated with stress such as reduced food intake, unaccustomed handling

or strenuous exercise (Todd, 1962).

Events which culminate in release of copper from the liver are not understood. To understand this process, liver fractionation studies have been done in sheep (Philip, 1973) and rats (Lal and Sourkes, 1971). Copper loading in both sheep and rats resulted in copper accumulation in mitochondria, the nuclear fraction, to a lesser extent in cytosol and least in microsomes. At high copper levels (>1,000 ppm) more copper was bound by the cytosol (Philip, 1973). Much significance was attached to the cytosolic copper binding due to occurrence of copper binding protein (Bremner and Marshall, 1974a,b). Copper binding occurred in three fractions with molecular weights as follows: fraction I, 75,000; fraction II, 35,000 and fraction III, 12,000. The third fraction represented the metallothionein and bound most copper in normal animals. In copper loading no consistent pattern of copper accumulation was noticed. In some calves more copper was bound in fraction I while in others, more was bound in fraction III. Fraction II, which represents the copper enzyme superoxide dismutase, did not participate in copper storage.

Different results were obtained by Philip (1973) in sheep. In sheep with normal hepatic copper concentration, most copper was bound by fraction I and less by fraction II. There was virtually no copper in the metallothionein fraction. Copper loading increased copper in fraction III. Saturation of binding sites on the metallothionein would result in free copper in the hepatocyte.

Histochemical studies in liver of copper loaded rats showed sequestration of excess copper in the lysosomes (McNary, 1963; Lindquist, 1968). Ishmael et al., (1971) observed a diffuse copper distribution in the hepatocyte in early stages whereas in later stages deposits observed near the bile canaliculi and in the Kupffer cells were in lysosomes. McNary (1963) recorded perinuclear copper deposits in the early stages before copper was sequestered in the lysosomes.

The mechanism of copper toxicity is not known. Evans (1973) observed that free ionic copper could produce death of hepatocytes by inhibiting enzymes. Histochemical studies have shown an overall reduction in activity of adenosine triphosphatase (ATPase) from the very early stages of copper loading in sheep (Ishmael et al., 1971). The inhibition became more pronounced as the hemolytic crisis approached. Biochemical studies have shown that copper has a specific inhibitory effect on microsomal ATPase (Peters and Walshe, 1966; Bowler and Duncan, 1970).

Copper accumulation was also accompanied by increased acid phosphatase activity in the liver of sheep (Ishmael et al., 1971). Acid phosphatase is located principally in the lysosomes (Gallagher, 1964). High concentrations of copper have been demonstrated in association with hepatocyte lysosomes in Wilson's disease (Goldfischer and Moskal, 1966) and in experimental copper poisoning in the rat (Goldfischer, 1967). Lindquist (1968) has shown that copper, sequestered within the lysosomes, initiates lipid peroxidation in lysosomal

membranes resulting in leakage of detrimental acid hydrolases.

Degenerative changes of hepatocytes were observed early in the course of experimental copper poisoning (Ishmael et al., 1971; Howell and Gopinath, 1977). Centrilobular necrosis of the parenchymal cells was an early observation and corresponded to areas of increased acid phosphatase activity and copper deposition (Ishmael et al., 1971). Swollen Kupffer cells were also seen early, and they exhibited increased acid phosphatase activity and copper deposition. Massive necrosis in the centrilobular and periportal areas was evident at hemolytic crisis and changes in Kupffer cells were more pronounced. Such massive necrosis may be associated with copper release into the bloodstream. Centrilobular necrosis observed in chronic copper poisoning was also observed in experimental acute copper poisoning (Ishmael et al., 1971) without elevated hepatic copper levels.

There was a loss of enzyme activity in necrotic areas with a concomitant increase of these enzymes in the plasma of copper poisoned sheep (Ishmael et al., 1971; Ishmael and Gopinath, 1972). Such enzymes included liver specific enzymes, glutamate dehydrogenase (GD), sorbitol dehydrogenase (SDH), serum glutamate oxaloacetate transaminase (SGOT) and arginase.

Hemolytic crisis. Passive accumulation of copper in the liver can take place for long periods with copper concentration increasing to high levels without evidence of toxicity. The toxic phase is an acute hemolytic crisis and is caused by or, at least, follows the release of copper into the blood

(Thompson and Todd, 1970). The clinical signs include jaundice, hemoglobinuria, loss of appetite and excessive thirst. The hemolysis is very rapid; the normal PCV of 35 to 40 percent falls to 10 percent or lower in less than two days.

Blood copper remains normal until one to two days before clinical signs appear, and then increases 5 to 20 times (McCosker, 1968; Todd and Thompson, 1963). Hemoglobin concentration falls rapidly over the next two to three days and methemoglobin (MHb) increases, peaks in one to two days and then falls again. The pattern is similar to that of blood copper. There is a dramatic decrease of reduced glutathione (GSH) and Heinz bodies are formed. These changes in MHb and GSH occur in other hemolytic conditions such as drug induced hemolysis and Wilson's disease in man and can be stimulated by repeated intravenous injections of small amounts of copper over two to four days (Todd and Thompson, 1964; Howell and Gopinath, 1977).

The mechanism of hemolytic anemia in chronic copper poisoning is not clearly understood. Low concentrations of ionic copper when suspended in isotonic saline cause hemolysis of erythrocytes (Lambin et al., 1951; Goldberg et al., 1956). Concentrations of 40 to 50 ug/100 ml cause hemolysis. If the cells are suspended in serum instead of saline, however, concentrations of copper up to 5,000 mg/100 ml do not cause hemolysis. Ionic copper inhibited ghost Na^+, K^+, Mg^{++}-ATPase (Bowler and Duncan, 1970) and this could lead to fragmentation of erythrocytes suspended in saline. However, the findings of

Thompson and Todd (1970) disproved this interpretation in natural cases of chronic copper poisoning. They found that a residue of liver homogenate from chronic poisoned sheep which remained after extraction with ether did not cause hemolysis of erythrocytes. It was possible that such copper was bound to proteins and amino acids and could not exert a direct effect on erythrocyte plasma membrane. Such copper penetrated the plasma membrane of the erythrocyte and caused MHb formation and GSH destruction. Ultrafiltrable copper from plasma of sheep during a hemolytic crisis represented a very small fraction of total whole blood copper and when the latter was elevated 5 to 7 times; the ultrafiltrable copper showed at most a two-fold increase. This would suggest that ionic copper was not directly responsible for the hemolysis (Goldberg et al., 1956; Todd and Thompson, 1963).

The mechanisms by which intracellular copper accumulation can lead to cell lysis are several. Probably the most important observation is the loss of GSH. GSH protects the cellular enzymes and hemoglobin from oxidation by being preferentially oxidized (Ganther et al., 1976). Loss of GSH resulted in membrane injury and lysis of erythrocytes (Kosower et al., 1969) and exposed hemoglobin to oxidative injury leading to methemoglobin and Heinz body formation as seen in the hemolytic phase of chronic copper poisoning (Jandl et al., 1960). GSH loss may be directly due to oxidation by copper as was observed in <u>in vitro</u> tests (Thompson and Todd, 1970; Fairbanks, 1967) or indirectly due to inhibition of pentose phosphate enzymes

responsible for maintaining adequate levels of GSH (Ganther et al., 1976).

In spite of the reported inhibition of glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) by copper in vitro (Deiss et al., 1970) and lowered G6PD in one Wilson's disease patient with hemolytic anemia (Passwell et al., 1970), no inhibition of these enzymes has been observed during hemolytic crisis in the sheep (Thompson and Todd, 1976). Inhibition of glutathione peroxidase (GSH-Px) would remove the preferrential oxidation of GSH and expose the cell to oxidative injury from peroxides (Ganther et al., 1976). This enzyme has not been investigated in chronic copper poisoning. Copper, like other hemolytic agents, is capable of generating peroxides which would also accelerate oxidation of GSH (Cohen and Hochstein, 1964).

Hemolysis in chronic copper poisoning is both extravascular and intravascular. The extravascular hemolysis is of minor importance and results from erythrophagocytosis which occurred in the reticuloendothelial system (RES) and mainly in the spleen (Soli and Nafstad, 1976).

Heinz bodies may accelerate the rate of red cell destruction (Jandl et al., 1960). They reduce red cell deformability resulting in sequestration in the spleen (Rifkind, 1965) and intravascular hemolysis (Lubin and Desforges, 1972). Heinz bodies, predominantly attached to the red cell membrane in copper poisoning, induce distortion (Soli and Nafstad, 1976).

Fragmentation of red blood cells has also been observed by the latter authors. Associated with this fragmentation are discontinuities in the membrane observed on electron micrographs.

Loss of the laminar structure associated with Heinz bodies (Soli and Nafstad, 1976) could have been due to the alteration in lipid composition observed by Szelenyi et al. (1972).

Direct oxidative injury to the cell membrane has been reported by Kosower et al., (1969). They demonstrated that chemical agents such as copper could induce free radical formation which would cause the membrane injury leading to hemolysis.

Clinical signs and pathologic anatomy. The signs of chronic copper poisoning have been described by many workers (Todd et al., 1962; Todd, 1969; Suveges et al., 1971). There are two recognisable phases in chronic copper poisoning; an asymptomatic phase when copper is accumulating in the liver and a toxic phase of an acute "hemolytic crisis". Suveges et al. (1971) recorded a loss of appetite, fatigue and difficult breathing at the onset of hemolytic crisis. Extensive hemolysis resulted in presence of free hemoglobin in plasma and urine. Methemoglobinemia occurs and may be the cause of the chocolate brown color of the blood and mucous membranes at the start of hemolysis. The mucous membranes become jaundiced, and this is more prominent if the animals survive for over two days. The temperature may or may not be elevated.

The illness progresses very rapidly and usually ends in death in two to four days. Mortality is high in animals that develop toxic symptoms. This seems to be especially true of British breeds of sheep. Merino sheep may survive more than one crisis (Marston, 1950).

Death from consumption of a large dose of copper is rapid and preceded by diarrhea, dyspnea and marked weakness (Ishmael and Gopinath, 1972).

Post-mortem findings include generalized icterus, yellow friable liver, and black kidneys which are congested with hemoglobin (Süveges et al., 1971; Todd et al., 1962). When illness is very acute and death occurs within 24 hours, the carcass and liver appear muddy brown rather than jaundiced from the hemoglobin and methemologlobin released during hemolysis. The liver may be enlarged and generally the cut surface is yellow. The spleen may be enlarged and congested; the parenchyma is deep brown to black. The bladder may contain dark urine. Excess pericardial fluid is often found (Todd, 1962).

Sheep dying from acute copper poisoning present an entirely different post-mortem picture. There is excessive fluid, possibly blood-stained, in the pleural, pericardial and peritoneal cavities. Edema of the lungs is common. In animals which die within 24 hours, subendocardial hemorrhages occur predominantly in the left ventricle. The liver may be dark and mottled. There may be edema and hemorrhage of the

gall-bladder. The kidneys are congested. Abomasal and duodenal mucosa are hemorrhagic (Ishmael et al., 1971).

Histopathology in experimental chronic copper poisoning has been described by Ishmael et al. (1971) and Suveges et al. (1971). Similar lesions have been produced in sheep by small doses of intravenously injected copper (Howell and Gopinath, 1977). Histopathologic changes develop quite early and are evident about three weeks after copper dosing starts. These include swelling of parenchyma cells, necrosis of isolated parenchyma cells, enlargement and vacuolation of parenchyma cell nuclei and swelling of isolated Kupffer cells. Necrosis is originally centrilobular and becomes periportal. Centrilobular necrosis is marked at the time of hemolytic crisis, and, in these areas, there is observed a collapse of reticulin framework. Nuclear enlargement and vacuolation are very prominent at the time of hemolytic crisis. Swollen Kupffer cells are noticeable after dosing starts. Rubeanic acid-positive copper granules are first seen as fine particles in the parenchymal cell cytoplasm and as dense granular deposits within swollen Kupffer cells. Bile pigment accumulation is observed in the canaliculi during and after hemolytic crisis. Post-hemolytic biopsy samples show fibroblastic proliferation in the portal areas. Periodic acid Schiff (PAS)-staining reveals a few glycogen granules in liver cells (Suveges et al., 1971). Soli and Nafstad (1976) described intrafollicular necrosis of the spleen which seemed to be associated

with obstruction of follicular arteries.

Lesions have been found in the kidneys prior to hemolysis and consist of an increase in size and number of intracytoplasmic granules in the renal tubular cells. These granules were PAS positive, diastase resistant, contained lipofuscin and were acid phosphatase rich. This suggested a lysosomal response to increased copper content (Gopinath et al., 1974). Coincident with onset of hemolysis, there was acute tubular degeneration and necrosis with loss of enzymes. The lumina of the Bowman's capsule and renal tubules may be distended with hemoglobin.

A status spongiosus (Gonatas, 1970) has been observed in the white matter of the brain and spinal cord of chronically poisoned sheep (Ishmael et al., 1971).

Biochemical changes in chronic copper poisoning.

Blood copper. There is no consensus on the changes in blood copper concentration during the period of copper accumulation. Thompson and Todd (1970) found that blood copper levels remained within the normal range until one or two days before the hemolytic crisis while Eden (1940) found a gradual rise in whole blood copper to approximately twice the initial levels followed by a very sharp "premortal" rise to about 10 times the initial value. Sutter et al. (1958) observed elevated serum copper concentrations as many as 35 days before death. He concluded that blood copper levels could be used for diagnosis. McCosker (1968b) recorded an

increase in whole blood copper to approximately twice normal levels two to three weeks before the crisis. MacPherson and Hemingway (1969), on the other hand, encountered only one case out of eight of elevated copper levels before clinical symptoms. Experience of other authors is that copper levels increased only during or immediately before the hemolytic crisis (Barden and Robertson, 1962; Todd and Thompson, 1963; Ishmael et al., 1972).

An initial increase was observed in plasma copper followed by an elevation of erythrocyte copper concentration (McCosker, 1968). The increase in plasma copper was mainly in the direct reacting fraction. Changes in blood copper fractions during hemolytic crisis resemble those seen after an intravenous dose of copper (Eden and Green, 1939; Todd and Thompson, 1964). Erythrocytes take up copper very slowly followed by a slow release while copper in plasma is cleared very rapidly.

Serum enzymes. There are marked increases in serum enzymes at the time of hemolytic crisis. These include SGOT, serum glutamate pyruvate transaminase (SGPT) and lactic dehydrogenase (LDH) (Todd, 1969). The activities of SGOT and LDH increased several weeks before the onset of hemolysis (Todd and Thompson, 1963; Ross, 1966; MacPherson and Hemingway, 1969). These enzymes might indicate general tissue damage (Boyd, 1962), but Ross (1966) observed that arginase, a liver specific enzyme, appeared in the serum before hemolytic crisis. This indicated that liver damage had

occurred. Ishmael et al. (1972) recorded increases in plasma arginase, sorbitol dehydrogenase and glutamate dehydrogenase, all of which are liver specific, within two weeks after copper dosing started. Ishmael et al. (1971) presented morphologic evidence of early liver damage during chronic copper supplementation. Reduced histochemical staining for glutamate dehydrogenase coincides with elevation of this enzyme in the plasma. Similar enzyme changes to those observed during the hemolytic crisis following chronic copper poisoning were produced by repeated intravenous injections of small doses of copper (Todd and Thompson, 1964; Howell and Gopinath, 1977) and were also accompanied by liver damage.

Sulfobromophthalein (BSP) excretion. Liver damage is also indicated by reduced clearance of BSP following start of copper administration. BSP clearance is markedly abnormal at the time of hemolysis (Ishmael et al., 1972). MacPherson and Hemingway (1969) observed increased BSP retention three weeks before death. Normal clearance was observed even after SGOT levels had increased. This test was therefore useful for diagnostic purposes only immediately prior to hemolytic crisis.

Bilirubin. Todd and Thompson (1963) recorded elevated serum bilirubin levels several days before clinical signs of chronic copper poisoning. Ishmael et al. (1972) observed a moderate increase in serum bilirubin during the first few weeks of copper administration. Levels returned to normal but rose sharply at the onset of the hemolytic crisis when most bilirubin was unconjugated. Prior to hemolysis, an

increase in bilirubin which was hepatocellular in origin occurred (McCosker, 1968).

Blood urea. Urea levels remained normal until hemolysis occurred, when very high levels were recorded (Todd and Thompson, 1963; Gopinath et al., 1974; Ishmael et al., 1972).

Ceruloplasmin. Ceruloplasmin activity increased at the time of copper-induced hemolytic crisis (Ishmael et al., 1972). The source of this increased activity is not known since only direct reacting copper increases in plasma at the time of hemolytic crisis (McCosker, 1968).

Reduced glutathione (GSH). GSH concentration falls markedly at the commencement of the hemolytic crisis to less than 10 percent of the pre-crisis levels (Todd, 1969). Although there is wide variation in GSH levels between individual sheep (Young et al., 1975), results indicate that hemolysis begins when the glutathione level falls to about 50 percent of its initial concentration. There is a corresponding drop in oxidized glutathione (GSSG) (Todd and Thompson, 1976). Since the lost GSH is not recovered as GSSG, Todd and Thompson (1976) suggested that GSSG is either lost into the plasma where it is rapidly destroyed, or it is oxidized further into lower oxidation states such as sulfinic, sulfonic and sulfoxide derivatives of GSH. Increased GSH levels are observed in the blood of animals recovering from anemia because immature erythrocytes have a greater GSH concentration (Thompson and Todd, 1976).

Erythrocyte pentose phosphate enzymes. Thompson and Todd (1976) found no increase in G6PD or GR prior to hemolytic crisis. Increase in the activities of the above enzymes following hemolytic crisis was due to the presence of immature erythrocytes which have higher activities of the enzymes.

Hematology.

Hematocrit, hemoglobin and methemoglobin. There was an increase in hematocrit (PCV) and hemoglobin concentration in the period before hemolytic crisis (Todd and Thompson, 1965; Todd and Thompson, 1963; Barden and Robertson, 1962; Ishmael et al., 1972). This increase in hematocrit and hemoglobin has been recorded even one to ten days before hemolysis (Todd and Thompson, 1963). Intravenous injections of copper salts were also followed immediately by a rise in PCV and hemoglobin concentration (Todd and Thompson, 1964; Ishmael and Gopinath, 1972). Several reasons for this increase in PCV and hemoglobin are given. Barden and Robertson (1962) attributed the increase to swelling of erythrocytes, while Todd and Thompson (1964) argued that increases in the above parameters were due to hemoconcentration.

The onset of hemolysis results in rapid reduction in both PCV and total hemoglobin. Increased presence of hemoglobin in plasma is observed (Todd and Thompson, 1963; Ishmael et al., 1972). This is accompanied by marked methemoglobinemia. PCV may drop as low as 6 to 10 percent, while hemoglobin concentrations as low as 3 g/dl have been recorded. Plasma hemoglobin and methemoglobin are cleared within two days of

hemolysis. Red cell counts followed the same pattern as PCV and hemoglobin (Ishmael et al., 1972).

Blood smears. During and immediately following hemolytic crisis, blood smears show fragmented red cells, anisocytosis, poikilocytosis and polychromasia. Heinz bodies were seen in supravitally stained smears. Recovery from hemolytic anemia was associated with reticulocytosis (Ishmael et al., 1972; Soli and Nafstad, 1976).

White blood cells (WBC). There was an increase in white blood count at onset of hemolysis with an increase in percentage of neutrophils (Ishmael et al., 1972).

Diagnosis and treatment.

Diagnosis. Signs of hemolysis and jaundice accompanied by high hepatic copper concentration are sufficient for a diagnosis of chronic copper poisoning (Todd et al., 1962). Elevated kidney copper concentration may be a better indicator since the levels remained low until hemolytic crisis occurred (Eden, 1940). However, the kidney copper levels are raised only temporarily, and if the animal survived three to four days, analysis of the kidneys may reveal normal or nearly normal levels.

According to MacPherson and Hemingway (1969) and Ross (1966) the most sensitive indicator of chronic copper poisoning was an elevation in SGOT. Measurement of this enzyme would insure a minimum period of three weeks prior to hemolytic crisis during which treatment could be instituted.

SGOT is not a liver specific enzyme (Boyd, 1962). Sorbitol

dehydrogenase (SDH), glutamate dehydrogenase (GD) and arginase are liver specific enzymes and they were elevated early during copper supplementation (Ishmael et al., 1972). Early elevation in arginase activity was observed by Ross (1966). These enzymes should be diagnostic of liver damage when they are increased.

BSP clearance test should aid in diagnosis of liver dysfunction but it was found to be useful only immediately prior to hemolytic crisis (MacPherson and Hemingway, 1969). Ishmael et al. (1972) observed a slight increase in retention of BSP a few weeks after copper supplementation but low clearance rates two weeks before hemolysis.

Liver biopsy for histologic studies and histochemical staining for copper would probably aid in early diagnosis. Ishmael et al. (1972) found definite morphologic changes early in the course of experimental copper poisoning. This was accompanied by marked copper deposition as demonstrated by the rubeanic acid staining technique.

Treatment. Following the observation by Dick (1954) that feeding excessive amounts of ammonium molybdate and sulfate limits copper storage in sheep's liver, Pierson and Aanes (1958) and Barden and Robertson (1962) have stopped sheep losses from chronic copper poisoning by inclusion of 50 mg ammonium molybdate and 1.5 g copper sulfate per kg feed. This treatment has also been used to lower copper levels in housed sheep (Ross, 1970). Harker (1976) has shown that addition of ammonium molybdate and copper sulfate to diets of housed lambs

CHAPTER II

CLINICAL, BIOCHEMICAL AND PATHOLOGIC CHANGES IN EXPERIMENTAL SUBACUTE AND CHRONIC COPPER POISONING IN SHEEP

Introduction

The initial phase of chronic copper poisoning in sheep is characterized by a variable period of copper accumulation in the liver during which time the animal is clinically normal (Todd, 1969). The second phase is acute, characterized by a hemolytic crisis and other clinical signs of illness. The sheep become icteric and there is hemoglobinemia and hemoglobinuria. Death may follow in 2 to 4 days or the animals may appear to recover only to undergo further hemolytic crises (Todd, 1962).

Prophylactic treatment with ammonium molybdate and copper sulfate has been effective in reducing losses, but when the animals develop clinical signs, there is no effective treatment (Todd et al., 1962). The reported increased incidence of chronic copper poisoning in housed sheep (Todd, 1972) and an increased exposure of grazing sheep to copper by the use of poultry waste or pig slurry with high copper content for fertilizing pastures (Hill, 1977) makes it important to have diagnostic procedures to identify the problem at an early stage, before extensive losses occur.

Copper poisoning in sheep causes liver dysfunction (Ishmael et al., 1971). Degenerative changes of the

reduces the risk of copper accumulation.

The problem still exists how to cure sheep in hemolytic crisis. Pierson and Aanes (1958) claimed some recoveries of clinical cases after supplementation with ammonium molybdate and sulfate. However, Todd et al. (1962) showed that such treatment did not guarantee a cure. They tried methylene blue to reduce the methemoglobinemia, injected ascorbic acid and tried raising GSH by injecting it into sheep. None of these treatments reduced the severity of signs or stopped the losses.

Of all the three chelating agents tested by MacPherson and Hemingway (1967) penicillamine induced the highest cupriesis. Sodium ethylenediamine tetra-acetate (NaEDTA) and dimercaprol (BAL) were less effective.

hepatocytes are notable early during the accumulation phase accompanied by release of enzymes from the liver into the blood (Ishmael et al., 1972). Reports on sulfobromophthalein (BSP) clearance during copper accumulation are variable. Ishmael et al., (1972) observed delayed clearance of this dye as early as 4 days after the beginning of copper administration while MacPherson and Hemingway (1969) recorded normal clearances until two to three weeks before death.

Liver function tests, though useful in determining extent of liver damage, cannot be the sole means of diagnosing copper poisoning. Reports of changes in blood copper concentration during development of copper poisoning agree only on the acute increase at the onset of hemolysis (Ishmael et al., 1972). Others also have reported blood copper increases early in the accumulation phase (Eden, 1940; Sutter et al., 1948; McCosker, 1968). An early rise in plasma ceruloplasmin copper has been described by McCosker (1968), and Ishmael et al. (1972) recorded a sharp increase in ceruloplasmin at the time sheep were undergoing a hemolytic crisis.

Kidney copper levels increase prior to hemolytic crisis (Gopinath et al., 1974) but urinary copper excretion during this period has not been measured.

The following studies were performed primarily to evaluate changes in hepatic function during chronic copper poisoning of sheep. Renal function was evaluated by measurement of plasma urea (BUN) and creatinine concentrations. Urinary and fecal copper excretion was measured in animals

placed in metabolism cages. Clinical signs and necropsy findings were recorded.

Materials and Methods

Chronic Copper Experiment

In the chronic copper feeding experiment, eight, six-month old wethers from the Cornell University Sheep Barns were housed in individual loose pens inside a barn. Each was fed 1 kg alfalfa pellets in a wooden box daily and given tap water ad lib in galvanized steel buckets. Water was changed daily.

After three days of adaptation to the environment and diet, the sheep were weighed. Three sheep were chosen randomly as controls and the other five were to be supplemented with copper. After a two week period of baseline data collection, the daily ration of the five experimental sheep was supplemented with 1 g $CuSO_4 \cdot 5H_2O$, an equivalent of 250 mg of copper per day. Initially the copper sulfate was ground into a powder and sprinkled on the pellets. Later, when the sheep avoided copper-containing pellets, the powder was dissolved in 10 ml distilled deionized water and the solution was sprinkled on the pellets. After 40 days, the daily copper sulfate dose was increased to 1.5 g, with an equivalent of 375 mg copper. This dose was continued until day 107 when the sheep which remained were supplemented with 2 g $CuSO_4 \cdot 5H_2O$ (500 mg copper) per day. Copper feeding was stopped when animals showed signs of

intravascular hemolysis. Details on the copper feeding schedule and animal data are shown in Tables 2-1 and 2-2.

Sheep were killed by exsanguination after barbiturate anesthesia at various times during the hemolytic crisis. Animals were weighed. Liver and kidney weights were recorded and samples were obtained for copper analysis and histopathology.

Subacute Copper Experiment

Four cross-bred rams, eight months of age were confined to metabolic cages and fed a ration of 1 kg alfalfa pellets and tap water. Copper analysis for the feed and water showed a daily intake of 12.8 ug.

Two of the rams, 114 and 133, were kept in aluminum cages while the other two, 129 and 116, were in wooden cages. The aluminum cages were designed to collect urine and feces from female sheep and therefore, an adaptation was made by fixing a plastic bag to the wire mesh on the floor of the cage and funneling urine into a plastic bottle. Feces were collected separately in a plastic tub. The wooden cages were designed to collect urine and feces separately from male sheep.

After an adjustment period, 24 hour urine and fecal collections were made during a ten day control period. Blood samples also were obtained for analysis.

Sheep 116 and 133 were drenched daily with 150 ml of a

1.0 percent copper sulfate solution in deionized distilled water. Drenching was done each day in the morning before feeding. Sheep 114 and 129 were untreated controls. Twenty-four hour collections of urine and feces were also made during the experimental period.

After four and eight days, respectively, sheep 133 and 116 became anorectic and depressed. Dosing with copper sulfate was stopped until the sheep resumed eating. Sheep 133 was killed during the hemolytic crisis while 116 was allowed to recover from the crisis and was killed sixteen days later. Details of these sheep, their diet and total copper consumption are shown in Table 2-3.

Necropsy studies were performed as described above for the chronic copper experiment.

Blood Samples

In the chronic copper experiment, venous blood was obtained weekly before signs appeared and daily when hemolysis was observed. Blood was obtained by jugular venipuncture and collected into heparinized vacuum tubes.

In the subacute copper experiment, sheep were bled twice weekly before clinical signs appeared and more frequently during the hemolytic crisis.

Blood was centrifuged[a] at 2,000 RPM for 15 minutes at

[a] Model J21B, Beckman Instruments, Inc., Fullerton, Calif. 92631.

2C. Plasma was transferred to copper-free vials and analyzed. Alternatively the samples were frozen at -20C until thawed for analysis.

Urine and Fecal Samples

In the study of subacute copper poisoning, 24 hour urine and fecal samples were collected as previoulsy described, mixed and duplicate aliquots were taken for copper analysis. These samples were frozen at -20C or processed immediately after collection.

Analytical Methods

Copper. Duplicate fecal, urine, liver and kidney samples were placed in copper-free acid washed crucibles, lyopholized[a], and dried at 100C to constant weight. Five ml of urine and 3 to 5 g aliquots of feces, kidney and liver were used.

After overnight ashing in a 500C muffle furnace[b], urine ash was dissolved in 2 ml of 4 N hydrochloric acid (HCl). Fecal, liver and kidney ash was dissolved in 5 ml of 4 N HCl and made up to 25 ml in a volumetric flask with deionized distilled water.

Plasma for copper determination was processed by the method of Olson and Hamlin (1969). One ml of plasma and 1 ml

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[a] Model 10-800, Virtis Freeze Drier, Virtis Co., Gardiner, N.Y. 12525.

[b] Hotpack Corp., Philadelphia, Pa. 19135.

of 20 percent trichloroacetic acid (TCA) were mixed and heated for 15 minutes in a 90C water bath. The mixture was revortexed and centrifuged at 2,000 RPM for 15 minutes in a refrigerated centrifuge.[a] The supernatant was taken for copper analysis. Copper concentration in duplicate samples was determined by atomic absorption spectrophotometry.[b]

Plasma analysis. Glutamate oxaloacetate transaminase (GOT) was determined by utilizing the following reaction:[c]

Aspartic acid + α-ketoglutaric acid $\xrightarrow{\text{GOT}}$ Oxaloacetic acid + glutamic acid.

In the reaction catalyzed by GOT, the oxaloacetic acid formed is reacted with 2, 4-dinitrophenylhydrazine (DNP) to form a colored hydrazone whose absorption spectrum is measured at 505 nm. To substrate containing 0.2 M DL-aspartate and 1.8 mM α-ketoglutarate, 0.1 ml plasma was added and incubated for one hour at 37C. DNP (20 mg/100 ml) was then added to the reaction mixture which was left at room temperature for 20 minutes before adding 5 ml of 0.4 N sodium hydroxide (NaOH). Optical density (OD) was read at 505 nm in a UV spectrophotometer.[d] Activity in Sigma-Frankel units per ml

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[a] Model J21B, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[b] Model 305B, Perkin Elmer, Norwalk, Conn. 06856.

[c] Sigma Technical Bulletin No. 505, Sigma Chemical Co., St. Louis, Mo. 63178.

[d] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

(S/F U/ml) was determined from a standard curve. If the GOT activity exceeded 216 S/F U/ml, the plasma was diluted 1 in 10 with distilled water and the assay repeated.

Alkaline phosphatase (AP) was determined by the Sigma methodology[a]. Hydrolysis of p-nitrophenyl phosphate yields p-nitrophenol and inorganic phosphate. When made alkaline by NaOH, p-nitrophenol is converted into a yellow complex whose absorbance is measured at 400 nm. The intensity of color formed is proportional to phosphatase activity.

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{Phosphatase}} \text{p-Nitrophenol} + H_3PO_4.$$

Plasma (0.1 ml) was incubated for 30 minutes at 37C with 0.5 ml of 0.4 percent p-nitrophenyl phosphate buffered with a glycine buffer consisting of 0.1 M glycine and 1 mM magnesium chloride, pH 10.5. Ten ml 0.02 N NaOH was added to stop the reaction and develop the color. Absorbance was read at 400 nm in a UV spectrophotometer[b]. Activity expressed as Sigma units per ml (SU/ml) was determined from a standard curve.

Sorbitol dehydrogenase (SDH) was measured according to Sigma methodology[c]. SDH catalyzes the reaction:

$$\text{D-Fructose} + \underset{\text{(high)}}{\text{NADH}} \xrightarrow{\text{SDH}} \text{D-Sorbitol} + \underset{\text{(low)}}{\text{NAD}}$$

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[a] Sigma Technical Bulletin No. 104, Sigma Chemical Co., St. Louis, Mo. 63178.

[b] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[c] Sigma Technical Bulletin No. 50UV, Sigma Chemical Co., St. Louis, Mo. 63178.

The rate of decrease of absorbance at 340 nm is used as a measure of SDH activity. The reaction mixture consisted of 0.2 mg reduced nicotinamide adenine dinucleotide (NADH), 2 ml of 0.1 M Tris (hydroxymethyl) amino methane, pH 7.5 and 0.5 ml plasma. The reaction was started by adding 0.5 ml of a 72 percent fructose solution and the decrease in OD was followed at 37C. Activity was expressed as SU/ml and was calculated using the equation:

$$SDH = \Delta \text{ OD/min} \times 58000 \times TC.$$

TC is a temperature correction factor calculated by subtracting 7 percent for every degree above 25C. (TC_{37} = 1 - 12 (.07) = .16.)

Plasma ceruloplasmin activity was measured according to the method of Houchin (1958) as modified by Rice (1962). The rate of oxidation of p-phenylenediamine (PPD) by copper oxidase (ceruloplasmin) is measured at 530 nm. The standardization to international units as made by Rice (1962) is based on similarity in absorption spectrum between Bandrowski's base, a pigment formed upon oxidation of PPD in aqueous ammoniacal solution with hydrogen peroxide, and the pigment formed in the PPD assay. The assay mixture of 1 ml freshly prepared 0.1 percent PPD[a] in acetate buffer pH 6.5 and 0.1 ml plasma was incubated for 15 minutes in a 37C water bath. The reaction was stopped by adding 5 ml of 0.02 percent sodium

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[a] p-Phenylenediamine Dihydrochloride, Eastman Kodak Co., Rochester, N.Y. 14650.

azide. Optical density was read at 530 in a UV spectrophotometer.[a] Under normal conditions a reagent blank was satisfactory but during the hemolytic crisis, due apparently to the red color of hemoglobin, it was necessary to have a plasma blank for each sample (see page 57). Activity was calculated in international units per liter (IU/L) as suggested by Rice (1962).

Total plasma bilirubin was measured by automated analysis[b] using the method adapted from Jendrassik and Grof (1938). The quantitative determination of bilirubin is based on the formation of colored azobilirubin when bilirubin reacts with diazotized sulfanilic acid. Color intensity of the alkaline azobilirubin is measured at 600 nm.

Plasma creatinine was determined using a Folin-Wu alkaline picrate reduction method modified for automated analysis.[b]

Blood urea nitrogen (BUN) was analyzed using a phenol-alkaline-hypochlorite method to measure the ammonia produced by the action of urease on the plasma.[c]

Prothrombin time (PT) in seconds was determined on plasma obtained from whole blood with sodium fluoride as anticoagulant using a Simplastin kit.[d] A PT test involves mixing of plasma

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[a] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[b] Methodology N-11b, Autoanalyzer I, Technicon Corp., Tarrytown, N.Y. 10591.

[c] Sigma Technical Bulletin No. 640, Sigma Chemical Co., St. Louis, Mo. 63178.

[d] General Diagnostics, Morris Plains, N.J. 07950.

sample with a tissue thromboplastin-calcium mixture, then determining the time in seconds required for the fibrin clot to form.

Plasma fibrinogen was determined using Dade reagents.[a] In the assay the enzyme thrombin converts the soluble plasma protein fibrinogen into its insoluble polymer, fibrin, which is then measured using a fibrometer.[b] Units are mg/dl.

Sulfobromophthalein (BSP) clearance. BSP clearance tests were performed on all the animals before copper feeding and repeated throughout the experiment (Table 2-4). A dose of 5 mg/kg body weight of BSP was administered as a 5 percent solution. A zero time sample was taken, the dose was injected into the right jugular vein and samples of blood were collected from the left jugular vein 2, 3, 5, 7 and 10 minutes later. Plasma (0.5 ml) was mixed with 3.5 ml 0.1 N NaOH and the optical density (OD) was read at 580 nm in a Beckman spectrophotometer.[c] Regression analysis of log BSP concentration versus time was done and half-time (T 1/2) in minutes was calculated from the equation for the regression line.

Serum analysis. In the subacute copper poisoning trial blood was collected from sheep at the time clinical signs developed. Serum creatinine, lactic dehydrogenase (LDH) and

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[a] Dade Diagnostics, Inc., Delaware Parkway, Miami, Fla. 33152.

[b] Scientific Products, McGraw Park, Ill. 60085.

[c] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

BUN were analyzed by automated analysis.[a]

Histopathology. Liver and kidney specimens were fixed in 10 percent buffered formalin. Tissues were embedded in paraffin, sectioned at 6 um and stained with hematoxylin and eosin (H&E) for routine histologic examination. Sections also were stained with Perls' Prussian Blue for iron, with rubeanic acid for copper (Uzman, 1956) and with the periodic acid Schiff (PAS) procedure following digestion with diastase.

Results

In the chronic copper feeding experiment, sheep 1456 reacted very early (Fig. 2-1). After this sheep had been on copper supplementation for 2 weeks, its plasma was icteric and clinical jaundice of the mucous membranes was noted. This was accompanied by abnormal liver function tests. GOT and bilirubin were elevated and BSP clearance was delayed (Fig. 2-1). However, this sheep continued to have a satisfactory appetite and copper feeding was continued until day 42 when hemoglobinuria and hemoglobinemia were noticed. Sheep 1450 and 1473 suffered two hemolytic crises each (Table 2-1). The first crisis in both these sheep occurred on day 54 after receiving 13.5 g copper and the second crises occurred on day 94 and 92, respectively. Sheep 371 underwent hemolytic crisis on day 114 after consuming 35.9 g copper. Sheep 373 survived the longest. It was found dead after being fed 44.9 g copper

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[a] Coulter Chemistry, Coulter Electronics, Inc., Hialea, Fla. 33010.

over 132 days. It never suffered an overt hemolytic crisis although death was assumed to be related to copper poisoning. The mean cumulative dose of copper to first hemolysis was 18.4 g.

Dosing of sheep 116 and 133 with 1.5 g copper sulfate as a 1 percent drench was accompanied by refusal of food, and copper supplementation was halted temporarily as shown in Figures 2-2 and 2-3. These sheep suffered hemolytic crises early after consuming 6.0 g and 3.75 g of copper, respectively. Sheep 116 appeared to recover completely from the hemolytic crisis on day 26 and was killed 16 days later. Sheep 133 suffered a crisis on day 24 and was killed in extremis on day 28.

At the time of hemolytic crisis the animals became dull and there was loss of appetite. This was accompanied by passing of dark red urine. Plasma samples at this time contained free hemoglobin (Fig. 2-4). Mucous membranes of sheep 1473, 133 and 116 became brownish on the first day of hemolysis. Later they became jaundiced and pale.

Animals recovering from hemolytic crisis showed a return of normal color to the urine and mucous membranes, and they regained normal appetites.

Although most sheep seemed to have maintained satisfactory bodily condition (Fig. 2-5), their weights (26.1 $\pm$ 1.0 kg, mean $\pm$ SEM) at post mortem were significantly lower ($p < 0.05$) than those recorded at the beginning of the experiment (29.4 $\pm$ 1.1 kg).

The mean post mortem weight of the control sheep (32.1

± 0.4 kg, mean ± SEM) did not differ from their initial mean weight of 31.7 ± 0.3 kg (mean ± SEM). The post mortem weight of control sheep was significantly higher than that of the copper poisoned sheep ($p < 0.01$) (Appendix Table A 1).

Plasma Copper

The plasma copper concentration of normal sheep was 0.84 ug/ml (± 0.02) during the experimental period with a range of 0.54 to 1.15 ug/ml. Plasma copper of the chronically poisoned sheep was significantly higher than controls during the hemolytic crisis ($p < 0.05$), (Fig. 2-6, Table 2-5). The increase in plasma copper was marked in 1473 during the second hemolytic crisis; the highest recorded value was 3.6 ug/ml. Plasma copper was not invariably elevated prior to the hemolytic crisis (Fig. 2-1). Plasma copper was elevated early in sheep 1456 and this was accompanied by abnormal liver function tests (Fig. 2-1).

The situation was different in the subacutely poisoned sheep which received 150 ml of 1 percent copper sulfate solution daily (Fig. 2-3). Increases in plasma copper were observed early in copper supplementation. Although plasma copper concentration decreased when copper dosing was suspended, plasma levels remained above those of matched control sheep (Fig. 2-3). Hemolytic crisis was accompanied by much higher levels of plasma copper (>2.00 ug/ml) than those attained in the chronically poisoned sheep fed copper over a longer period of time.

Ceruloplasmin

The activity of this enzyme fluctuated over the copper feeding period but there was no difference between control and copper poisoned sheep during most of the feeding period (Fig. 2-1 and 2-6). Ceruloplasmin in the control sheep ranged from 14.7 - 45.0 IU/L over the experimental period. In chronic copper feeding, ceruloplasmin activity two weeks before hemolytic crisis was 23.1 ± 1.15 IU/L compared to 27.9 ± 0.97 IU/L (mean ± SEM) in control sheep, a difference not significant at the 5 percent level. At the time of the hemolytic crisis, the mean ceruloplasmin levels of poisoned sheep was 22.3 ± 1.8 IU/L, a signficiant difference ($p < 0.05$).

Choice of blanks significantly affected the apparent ceruloplasmin activity in hemolyzed plasma samples (Table 2-6). Ceruloplasmin activity obtained using a standard reagent blank was 44.9 ± 6.7 and 20.2 ± 2.6 IU/L (mean ± SEM) ($p < 0.01$) when the proper plasma bank was used.

Copper in Urine and Feces

Fecal and urinary copper excretion studies were performed only on the subacutely poisoned sheep. There was in increase in urinary copper excretion which corresponded to peaks in plasma copper when sheep were dosed with 150 ml of 1 percent copper sulfate (Fig. 2-3). Copper excretion in urine of 116 and 133 before copper supplementation was 0.05 ± 0.004 mg (mean ± SEM) copper for 24 hours as compared to 1.00 ± 0.284 mg/24 hr during the copper feeding period. This increase

could not be attributed to decreased urine output. Urine output in copper poisoned sheep (909 ± 122.4 ml) during the copper feeding period was significantly higher ($p < 0.05$) than output in control sheep (701 ± 39.7 ml).

Fecal copper excretion increased with copper dosing (Fig. 2-3). During the copper feeding period approximately 17.6 percent of the ingested copper was apparently absorbed and 82.4 percent excreted in feces. Of the absorbed copper, a maximum of 0.43 percent was in the urine and the rest was either stored or probably excreted through the bile into feces.

Glutamate Oxaloacetate Transaminase (GOT)

Plasma GOT activity in the control sheep during the experimental period ranged from 35 to 85 S/F U/ml with a mean of 56.3 ± 1.4 S/F U/ml. GOT activity in the individual sheep is shown in Figure 2-1 for the chronic copper feeding experiment and in Figure 2-2, for the subacute copper experiment.

GOT activity was elevated in sheep 1456 a fortnight after copper feeding began (216 S/F U/ml). High levels of this enzyme were sustained until hemolytic crisis occurred on day 42 (Fig. 2-1). The highest activity (1170 S/F U/ml) was recorded 21 days before frank hemolysis was noted, however.

GOT activity in the three other chronically poisoned sheep (1450, 1473, 371) remained normal (Fig. 2-1) during most of the copper feeding. Significant mean increases occurred

during the period prior to the hemolytic crisis but were most marked during the crisis. The mean activity of all the copper poisoned sheep during hemolytic crisis (353 ± 37.5) was significantly increased ($p < 0.01$) over that of the control sheep (56.3 ± 1.4) as shown in Figure 2-6 and Table 2-5. The acitivity of GOT varied between sheep. In sheep 1450, only moderate elevations occurred during the two crisis periods (190 and 285 S/F U/ml). Activity in 1473 during similar hemolytic periods was more marked (450 and 660 S/F U/ml respectively).

Figure 2-2 shows plasma GOT activity in the subacute copper experiment. There was an early rise in GOT levels of both sheep 133 and 116, noted first on days 4 and 8 of the study, respectively. The activity of this enzyme dropped slightly when copper dosing was halted temporarily but was markedly elevated during the hemolytic crisis.

Alkaline Phosphatase (AP)

There was no significant change in plasma AP activity in copper poisoned sheep during copper accumulation (Figs. 2-1, 2-2). The AP activity in control sheep was 2.3 ± 0.13 SU/ml (1.2 to 5.8). Activity of this enzyme was normal in copper poisoned sheep two weeks prior to hemolysis (2.11 ± 0.25 SU/ml) and during the hemolytic crisis (2.11 ± 0.12 SU/ml) (Fig. 2-6 and Table 2-5).

Sorbitol Dehydrogenase (SDH)

The activity of this enzyme was analyzed during the hemolytic crisis in chronically poisoned sheep. Figure 2-7 shows

the markedly elevated activity in copper poisoned sheep (13,873 ± 8,839 SU/ml) as opposed to activity in control sheep (505 ± 52 SU/ml), indicating significant hepatocellular dysfunction.

Lactate Dehydrogenase (LDH)

This enzyme was also analyzed during the hemolytic crisis period and is shown in Figure 2-7. LDH activity in hemolyzing sheep (577 ± 68 IU/L) was increased over that of control sheep (106 ± 5.9 IU/L) during the same period.

Plasma Proteins

There was no difference between copper poisoned and control sheep in either plasma albumin or fibrinogen (Fig. 2-8). Prothrombin, measured as the prothrombin time, also was normal (Fig. 2-8). These findings suggest the protein synthetic functions of the liver are maintained even during the terminal hemolytic crisis.

Total Plasma Bilirubin

Plasma bilirubin concentrations of individual sheep are shown in Figure 2-1. In the chronic copper experiment, bilirubin levels remained within normal limits throughout most of the feeding period in all sheep except 1456. Plasma bilirubin above 1.0 mg/dl was recorded 2 weeks after the beginning of copper feeding in the latter sheep and corresponded to the increased GOT activity noted earlier. The mean (±SEM) of 0.29 ± 0.06 mg/dl in copper poisoned sheep two weeks before

hemolytic crisis was significantly different from the 0.11 ± 0.01 mg/dl (0.05 to 0.20) of the control sheep. However, this bilirubin level cannot be considered significantly elevated (Fig. 2-6). The bilirubin level during the hemolytic crisis, however, was 0.96 ± 0.14 mg/dl (0.2 to 2.3) and was significantly elevated over that of control sheep ($p < 0.01$, Table 2-5).

In the subacute study, bilirubin concentrations increased three to five days before the hemolytic crisis (Fig. 2-2) with levels ranging from 1.7 to 3.35 mg/dl. Further increases were recorded after the hemolytic crisis. Maximum bilirubin of 4.6 mg/dl was recorded in sheep 133 while the highest concentration attained in sheep 116 was 3.7 mg/dl.

Sulfobromophthalein (BSP) Clearance

BSP clearance rates are shown in Table 2-4 and Figure 2-1. After two weeks on copper supplementation sheep 1456 had a delayed clearance time (11.6 min.) which was also delayed during the hemolytic crisis (11.1 min.). In all other sheep, BSP clearance was normal except during periods of hemolysis when the T 1/2 ranged from 6.1 to 11.1 minutes.

In the subacute copper experiment delayed excretion of BSP was observed early. Five days after the start of copper feeding, T 1/2 was 5.3 minutes in sheep 133 (Table 2-4). Significant delay in clearance occurred during the hemolytic crisis. In sheep 116 which survived the crisis, BSP clearance was delayed as late as 16 days after the hemolytic crisis (4.7 minutes).

Blood Urea Nitrogen (BUN)

BUN levels in control sheep were 16.3 ± 1.98 mg/dl (3.5 to 48.5) over the experimental period. BUN concentration in chronically poisoned sheep did not change even during the hemolytic crisis when levels ranged from 3.4 to 34 mg/dl with a mean of 15.1 ± 3.4 mg/dl. The highest recorded BUN concentration (47.7 mg/dl) was in sheep 371 two days after hemolytic crisis (Appendix Table A 2).

Serum analysis in sheep 133 which was dosed with a 1 percent solution of copper sulfate showed a high level of 170 mg/dl three days after the start of the hemolytic crisis.

Creatinine

Plasma levels of creatinine followed the same pattern as the BUN in the chronically poisoned sheep. Creatinine level in control sheep was 0.97 ± 0.12 mg/dl (0.6 to 1.2). Mean creatinine concentration of copper poisoned sheep during hemolytic crisis was 1.04 ± 0.23 and the range was 0.80 to 1.75 mg/dl.

The creatinine level in sheep 133 on the subacute copper experiment was 8.8 mg/dl three days following onset of the hemolytic crisis, a value markedly elevated above the normal of 0.7 mg/dl. The creatinine and BUN elevation in 133 indicated significantly impaired renal function following the hemolytic crisis.

Necropsy

Macroscopic Examination. Post mortem examinations were

performed on three control sheep, one subacutely poisoned and all five chronically poisoned sheep. Two sheep (1473 and 133), killed shortly after the beginning of the hemolytic crisis, had similar post mortem lesions. They had pale mucous membranes and the serosal surfaces also appeared pale. The kidneys were swollen and the cortex and medulla were dark reddish black. The urinary bladder was filled with dark red urine. The blood was thin and watery. The livers were tan yellow and the gall bladders were distended by thick, dark green bile. Sheep 1456, 1450 and 371 were killed four to six days after the appearance of signs of intravascular hemolysis. The macroscopic lesions were similar to those of the sheep killed during the hemolytic crisis. Urine in the bladder was red tinged. The livers were tan.

Sheep 373 which died after 132 days without developing clinical signs of intravascular hemolysis had hemorrhages in the mucosa of duodenum and colon. The thoracic and peritoneal cavities contained an excess amount of clear, straw-colored fluid.

Microscopic Examination.

Liver. All livers from the chronic copper feeding experiment including the controls had a mild bile duct hyperplasia. There was multifocal necrosis of the hepatocytes (Fig. 2-9b) which was most marked in the centrilobular areas. Neutrophils frequently invaded the focal areas of necrosis. The dying hepatocytes contained brown granular pigment which was PAS-positive, diastase resistant (Fig. 2-9c), stained

negatively for iron with Perls' Prussian Blue and positively with rubeanic acid (Fig. 2-9d). Enlarged Kupffer cells were present throughout the lobules and in some cases these macrophages concentrated around the central veins (Fig. 2-9b). They were filled with the brownish granular pigment which also was PAS-positive and diastase resistant, stained negatively for iron, and was positive with rubeanic acid stain. In some cases these pigment laden macrophages were observed in portal areas.

Kidney. Proximal tubules from the kidneys of sheep 133 and 1473 showed marked epithelial necrosis and desquamation (Fig. 2-10a). The lumens of the tubules were sometimes filled with necrotic debris. Dilated cortical and medullary tubules sometimes contained homogeneous eosinophilic casts (Fig. 2-10b). The epithelium of the proximal convoluted tubules contained large amounts of brown granular pigment which stained for hemosiderin with Perls' Prussian Blue (Fig. 2-10c). In sheep 133, there was interstitial fibrosis in the cortex accompanied by a mononuclear infiltration. The proximal tubular epithelium also contained rubeanic acid positive copper granules (Fig. 2-10d).

Discussion

The chronically poisoned sheep given copper sulfate in their feed showed significant variation in resistance. One

sheep, 1456, developed signs of hepatic dysfunction as early as two weeks after start of supplementation but did not have a hemolytic crisis until it had been fed copper for 42 days. This sheep appeared to be recovering clinically from the mild attack when it was killed. The other chronically poisoned sheep underwent mild hemolytic crises. Sheep 1473 and 1450 went through the first crisis and probably would have survived the second crisis if they had not been killed. Sheep 371 received a total of 35.9 g copper before its first crisis. It was killed while recovering from this crisis. Sheep 373 was found dead on day 132 after receiving 44.9 g of copper. The mean total copper dose to first hemolytic crisis in the chronic experiment was 18.4 g.

The two sheep which were drenched with a solution of copper sulfate suffered very severe hemolytic attacks after 24 and 26 days of copper dosing after consuming a mean of 4.9 g copper. Todd (1962) observed that individual susceptibility to relatively large doses was quite variable. Individual susceptibility is also evident from the work of Gopinath and Howell (1975) where four of eight sheep recovered from hemolytic crises and some suffered several attacks.

The form in which copper is administered can influence the degree of toxicity (Chapman et al., 1962). In addition the sheep which were dosed with 150 ml of 1 percent copper sulfate solution were handled daily adding another stress which could contribute to the observed early hemolytic crisis attacks (Todd, 1969).

The clinical signs recorded here were similar to those reported by Gopinath and Howell (1975) in experimental chronic copper poisoning and by Todd (1969) in natural outbreaks.

The post mortem findings and histopathology in liver and kidney were described by Gopinath and Howell (1975), Ishmael et al. (1971) and Gopinath et al. (1974). The lesions observed in sheep 373 which died acutely resembled those of acute copper toxicity described by Ishmael and Gopinath (1972).

Plasma copper changes in the chronically poisoned sheep resemble those described by MacPherson and Hemingway (1969). One sheep (1456) had moderately elevated plasma copper levels (1.51 ug/ml) several days before the hemolytic crisis. In the latter animal and sheep 1450, the crises were so mild that no elevated plasma copper levels were detectable when hemolysis was noted. The highest recorded plasma copper was 3.58 ug/ml in sheep 1473. As regards the nearly normal copper levels recorded during the crises, time of sampling for copper analysis is critical, especially when the crisis is as mild as those observed in sheep 1450 and 1456. Todd and Thompson (1964) and Eden and Green (1939) showed that injected copper is rapidly cleared from plasma. This rapid clearance was seen in sheep 1473 whose plasma copper level was very high the day before crisis but was only marginally elevated on the crisis day.

The two sheep dosed with 1 percent $CuSO_4 \cdot 5H_2O$ solution had plasma copper levels elevated as early as 4 days which continued to rise until the hemolytic crisis. This difference

may be because these sheep were dosed with a copper sulfate solution before being offered food in the morning. Eden (1943) showed that when a solution containing 200 mg copper was dried on feed, it did not affect rabbits for 48 days but was fatal within an hour when given in a single dose through a stomach tube.

Blood copper changes in chronic copper poisoning have been reported by several workers. Elevated copper levels were found immediately before hemolytic crisis by Barden and Robertson (1962), Todd and Thompson (1963) and Ishmael et al., (1972). Elevated copper levels have been reported several days before hemolytic crisis (Eden, 1940; Sutter et al., 1958; McCosker, 1968). Sutter et al. (1958), who observed elevated levels as early as 35 days before hemolytic crisis, suggested that blood copper levels could be used for diagnosis of chronic copper poisoning. The present work demonstrated, however, that use of plasma copper levels for diagnosis of chronic copper is precluded by occurrence of normal plasma copper levels until the hemolytic crisis. This would be expected in field cases where low amounts of copper are consumed over long periods before the sheep have acute hemolytic episodes. MacPherson and Hemingway (1969) and Ishmael et al. (1972) had similar observations in experimental copper poisoning.

Ceruloplasmin values remained normal with copper intake. During the hemolytic crisis, use of a reagent blank in the assay resulted in higher apparent ceruloplasmin activity than use of plasma blank. This supports observations by Ravin

(1961). The present results agree with those of Milne and Weswig (1968) who found that ceruloplasmin activity remained normal with increased copper intake when rats were not copper deficient. However, Ishmael et al. (1972) found elevated levels during hemolytic crisis. It is likely that they overlooked the presence of hemoglobin in hemolyzed samples. A human patient whose burn wounds were cauterized with copper sulfate subsequently suffered a hemolytic crisis accompanied by increased ceruloplasmin activity (Holtzman et al., 1966). The reason for increased ceruloplasmin activity was not evident, but the increase was considered to result from the higher body copper. Ceruloplasmin activity is a good indicator of copper deficiency (Lorentz and Gibb, 1975) but is not affected when an animal is in positive copper balance. McCosker (1968) observed that increased copper levels during a hemolytic crisis were attributable to direct reacting copper and not to copper in ceruloplasmin.

Urine copper excretion was increased early in the two sheep which were dosed with 1 percent $CuSO_4 \cdot 5H_2O$ solution. Peak urinary copper excretion coincided with plasma copper peaks. Urinary copper increases during hemolytic crises and levels may be as high as 2500 ug/ml (Todd and Thompson, 1964). Gopinath et al. (1974) found that kidney copper levels were elevated before a hemolytic crisis. This indicated that high levels of circulating copper were trapped in the kidney in addition to being excreted as observed in the present experiment. The two sheep used in this study had very high kidney

copper concentrations (See Chapter III of thesis). Sheep 133 had 173 ug/g while sheep 116 had 671 ug/g dry matter.

GOT activity in the chronic copper experiment remained normal in 4 of 5 poisoned sheep before hemolytic crisis (Fig. 2-6). At this time GOT activity varied from 190 to 700 S/F U/ml. The sheep with low activity (1450) suffered a mild hemolytic attack, and its other plasma parameters were not significantly elevated. Sheep 1456 had elevated GOT activity after 2 weeks of copper feeding. This indicated a liver dysfunction which was confirmed by increased BSP clearance time (T 1/2 was 11.1 minutes). The activity of this enzyme remained high in 1456 until hemolytic crisis was evident as typified by hemoglobinuria and hemoglobinemia. Sheep 133 and 116 which were given 1.5 g $CuSO_4 \cdot 5H_2O$/day as a drench had increased plasma GOT as early as five days post-dosing.

The observation that GOT was elevated several days before clinical symptoms of chronic copper poisoning were noticed (Todd and Thompson, 1963, 1965; MacPherson and Hemingway, 1969; Ross, 1966) suggested its use in diagnosis of an imminent hemolytic crisis. The findings of Ishmael et al., (1971) which revealed histological changes in hepatic parenchyma very early during copper accumulation were used to support the use of GOT for diagnostic purposes in chronic copper poisoning. They recognized that, although GOT analysis was not liver specific (Boyd, 1962), in known cases of copper exposure the enzyme could be used to measure the damage caused

by copper accumulation. The present work indicated that this cannot be a rule as most of the sheep in the chronic copper feeding experiment did not have elevated GOT until the time of hemolysis. One sheep (373) died acutely after consuming 44.9 gm of copper without elevation of GOT.

LDH has also been recommended as a diagnostic test for copper poisoning (Todd, 1969). This enzyme was found elevated during hemolytic crisis.

Sorbitol dehydrogenase (SDH), a liver specific enzyme, (Ishmael et al., 1972) was analyzed during hemolytic crisis and levels were markedly elevated at this time.

BSP clearance is a good indicator of liver hepatic excretion function (Kaneko and Cornelius, 1970). When GOT is marginally elevated, BSP test would prove useful in deciding if there is abnormal liver function. BSP clearance used together with GOT would be useful in cases where source of GOT was questionable. During the hemolytic crisis BSP clearance time was always increased. The present results agreed with those of Ishmael et al. (1972), who observed delayed BSP clearance as early as four days after copper feeding started. This was observed in sheep dosed with 1.5 g $CuSO_4 \cdot 5H_2O$/day. However, MacPherson and Hemingway (1969) frequently found normal BSP clearance after elevation of GOT but clearance was always delayed two weeks before death.

Prothrombin time, plasma fibrinogen, total protein and albumin were normal during hemolytic crisis. These parameters are used as measure of hepatic protein synthesis. All these

proteins are synthesized in the liver (Kaneko and Cornelius, 1970) and in event of acute and extensive liver damage, the plasma levels would be lowered. The fact that they are normal during the hemolytic crisis of chronic copper poisoning in the present work can be interpreted as meaning that a great portion of the liver is still functional at this time.

Plasma bilirubin levels were only slightly elevated in the sheep supplemented with copper in their feed. The mean level during hemolytic crisis was 0.96 ± 0.14 mg/dl (Table 2-5 and Fig. 2-6). The highest value of 3.0 mg/dl was recorded in sheep 133 which had a severe hemolytic attack after 24 days of copper feeding. Bilirubin concentrations were increased in those two sheep (116 and 133) which were dosed with 1.5 g in solution of copper sulfate/day. This increase was observed as early as five days after copper drenching was commenced. Levels remained above normal and were markedly increased during the crisis.

There was no clinically significant increase in total plasma bilirubin in chronically poisoned sheep until hemolytic crisis when levels above 1.0 mg/dl were recorded (Fig. 2-6). The exception was sheep 1456 whose bilirubin rose early in conjunction with increased GOT activity and BSP retention. Todd and Thompson (1963) reported increased levels before hemolytic crisis, but Ishmael et al. (1972), reported increased bilirubin levels only during the hemolytic crisis.

Plasma alkaline phosphatase activities remained normal in all sheep examined (Fig. 2-6). This enzyme is only expected

to be elevated in liver disease if there is a bile duct obstruction (Kaneko and Cornelius, 1970).

The BUN and creatinine were not increased in sheep chronically poisoned with copper. In the subacute copper experiment, high levels of BUN and creatinine were recorded during the hemolytic crisis. They were especially marked in sheep 133. These findings agree with those of Gopinath and Howell (1975) who found that BUN was never significantly elevated in sheep which recovered from a crisis while it was invariably increased in those sheep which later died.

Marston (1950) suggested that kidney failure was the cause of death due to blockage of the kidney by hemoglobin casts which resulted in uremia. Hemoglobinemic nephrosis as the sole cause of kidney failure was refuted by Todd and Thompson (1964) who showed that multiple injections of copper produced severe hemolysis but did not produce severe uremia and were followed by recovery of affected sheep. Eden and Green (1940) and McCosker (1968) observed that kidney failure could be due to acute toxic effects of copper. Studies of Lowe (1966) on rabbits showed that hemoglobinuria alone could not produce morphologic changes but administration of a small dose of nephrotoxic agent, which by itself would not produce damage, when combined with hemoglobinuria produced severe necrosis of the tubules. In the chronically poisoned sheep, plasma copper was never markedly elevated and kidney copper levels were not significantly elevated above those of normal

sheep (see Chapter III of thesis) possibly explaining why these sheep did not show impaired kidney function.

Summary

Drenching with 1.5 g $CuSO_4 \cdot 5H_2O$/day as a 1 percent solution and associated stress of handling produced a subacute copper poisoning in two sheep. Anorexia, hemoglobinuria, hemoglobinemia and jaundice were recorded in these sheep.

Supplementary copper in the diet in doses from 250 to 500 mg copper produced a mild disease. All sheep except 373, suffered mild hemolytic crises from which they were recovering when killed. Two sheep went through two crises before they were killed. Sheep 373 died acutely after receiving a cumulative dose of 44.9 g.

In chronically poisoned sheep, plasma copper was significantly increased only during the hemolytic crisis in 4 out of 5 sheep. Liver and kidney function tests remained normal until the crisis when the former were significantly elevated above normal. Their use in diagnosis of subclinical chronic copper poisoning would not be useful in cases which would later undergo mild crises. Plasma BUN and creatinine levels seem to have prognostic significance in predicting severity of a crisis.

Histopathologic studies demonstrated centrolobular liver necrosis with enlarged Kupffer cells filled with copper pigments. Similar macrophages were present in portal areas.

These changes support the conclusions drawn from the plasma biochemical observations.

Severe hemoglobinemic nephrosis was observed in sub-acutely poisoned sheep to explain the only abnormal kidney function tests.

Table 2-1. Details of Sheep in the Chronic Copper Feeding Experiment.

Animal ID	Status	Days to Hemolysis		Comments
		1st crisis	2nd crisis	
1450	Copper fed	54	94	Killed 3 days following onset of second hemolytic crisis.
1456	Copper fed	42	--	Killed 5th day of hemolytic crisis.
1473	Copper fed	54	92	Killed on 1st day of 2nd crisis.
371	Copper fed	114	--	Killed 4 days after crisis.
373	Copper fed	--	--	Found dead on day 132.
1451	Control	--	--	--
1454	Control	--	--	--
1461	Control	--	--	--

Table 2-2. Number of Days Fed Copper in Chronic Copper Experiment.

Daily Copper Dose	Sheep ID 1456	1450	1473	371	373[a]
250 mg	42	54[b]	54	56	56
375 mg	-	34	31	50	50
500 mg	-	-	-	8	26
Cummulative copper consumption (g)	10.5	25.9	24.8	35.9	44.9

[a] Sheep 373 was found dead on day 132 without previous overt signs of intravascular hemolysis.

[b] Copper supplementation stopped temporarily when 1450 and 1473 developed first mild hemolytic crisis on Day 54. Copper feeding was resumed for both sheep on day 61 of experiment.

Table 2-3. Details of Sheep in the Subacute Copper Poisoning Experiment.

Sheep No.	Status	Total Copper Consumption (gm)	Days to Hemolysis	Days to Death[a]
116	Copper fed	6.0	26	42
133	Copper fed	3.75	24	28
114	Control	-	-	36
129	Control	-	-	42

[a] Animals were euthanized using T61.

Table 2-4. BSP T 1/2 (minutes).

Subacute Copper Experiment

Day	Copper Poisoned		Control	
	116	133	114	129
0	1.6	2.1	1.2	2.3
5	-	5.3	2.1	-
26	4.4	16.0	1.9	1.9
42	4.7	*	*	2.2
Mean ± SEM			1.93 ± 0.16	

Chronic Copper Experiment

Day	Copper Poisoned Sheep					Control Sheep		
	1450	1456	371	373	1473	1451	1454	1461
0	2.3	1.0	1.9	1.3	1.2	2.1	2.1	1.7
16	-	11.6	-	-	-	2.0	-	-
43	-	11.1	-	-	2.2	2.3	-	-
55	2.5	*	2.3	1.9	9.4	*	1.9	-
97	8.0		3.7	1.9	*		1.9	2.9
113	*		6.1	3.7			2.9	2.9
Mean ± SEM						2.27 ± .46 (.15)		

* Sheep dead.

Table 2-5. Clinical Biochemistry of Control Sheep and of Copper Poisoned Sheep During Two Week Period Preceding Hemolytic Crisis and During Hemolytic Crisis (mean ± SEM).

Parameter	Control Sheep	Copper Poisoned Sheep: Pre-crisis Period	Copper Poisoned Sheep: Period of Hemolytic Crisis
GOT (S/F U/ml)	56.3 ± 1.4(73)[a] (35-88)[b]	128 ± 7.9(11) p <0.01[c]	353 ± 37.5(20) p <0.01
Alkaline Phosphatase (SU/ml)	2.3 ± 0.13(67) (1.2-5.8)	2.11 ± 0.25(20) ns	2.11 ± 0.12(18) ns
Ceruloplasmin (IU/L)	27.9 ± 0.97(71) (14.7-45)	23.1 ± 1.15(11) ns	22.3 ± 1.8(20) p <0.05
Total Bilirubin (mg/dl)	0.11 ± 0.004(71) (0.05-0.20)	0.29 ± 0.06(15) p <0.01	0.96 ± 0.14(18) p <0.01
Copper (ug/ml)	0.84 ± 0.02(69) (0.54-1.15)	0.82 ± 0.07(11) ns	1.25 ± 0.13(21) p <0.01
BUN (mg/dl)	16.34 ± 1.98(68) (3.5-48.5)	15.13 ± 2.29(6) ns	15.13 ± 3.38(20) ns
Creatinine (mg/dl)	0.97 ± 0.119(67) (0.6-1.2)	1.015 ± 0.38(6) ns	1.038 ± 0.23(20) ns

[a] Number of analyses.

[b] Range.

[c] Student's t test comparing controls with copper poisoned sheep.

Table 2-6. Effect of Blank in Ceruloplasmin Assay of Hemolyzed Samples.

	Ceruloplasmin (IU/L)	
	No. of Observations	Mean + SEM
Reagent Blank	13	44.9 ± 6.7
Plasma Blank	13	20.2 ± 2.6
Significance (Student's t test)		$p < 0.01$

Figure 2-1. Liver function tests, plasma copper and plasma ceruloplasmin values during chronic copper feeding experiment. Dotted line indicates development of intravascular hemolysis. Rectangle (▭) represents the range seen in the control sheep.

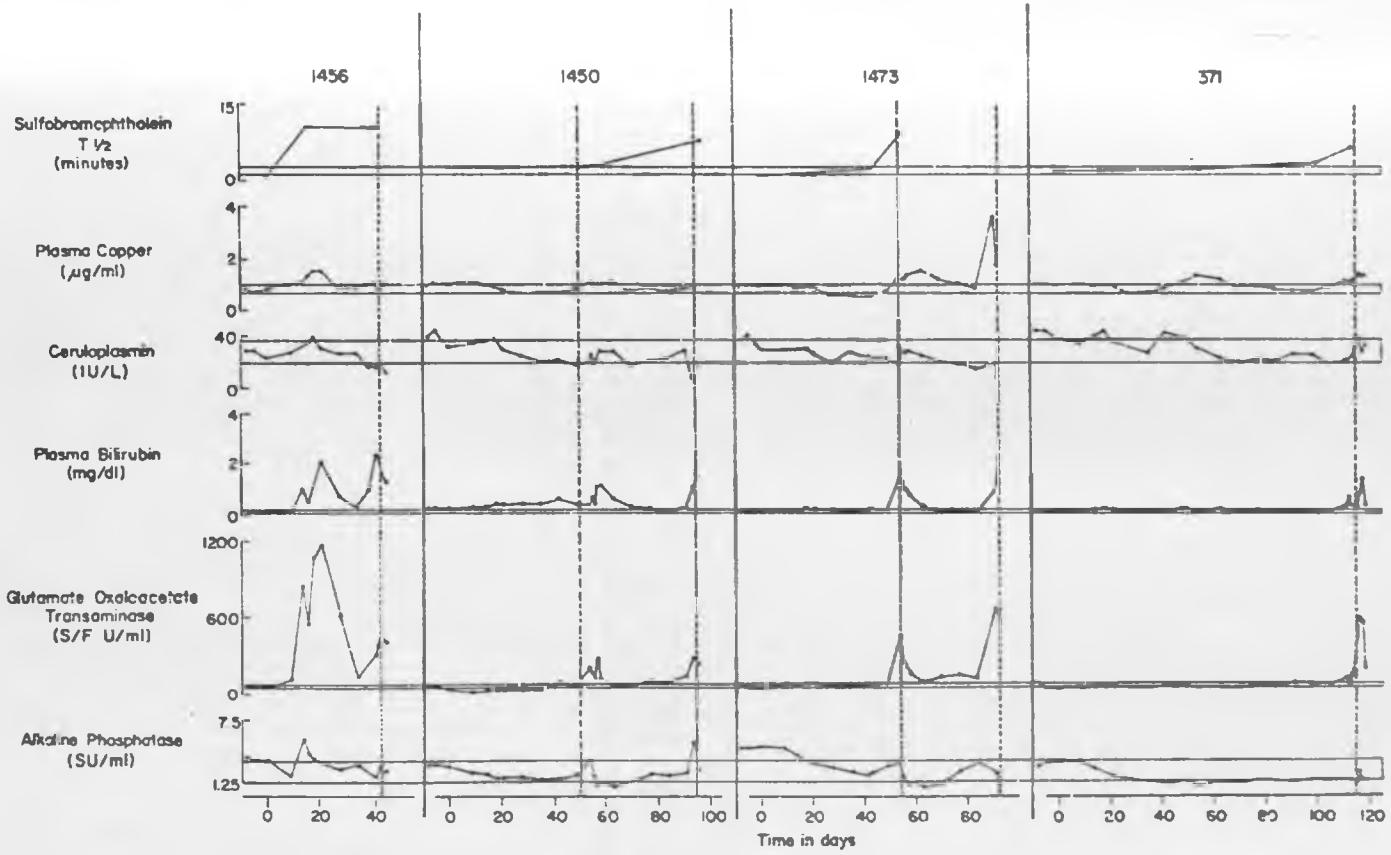

1456
1450
1473
371
Sulfobromophtholein T ½ (minutes)
15
0
Plasma Copper (µg/ml)
4
2
0
Ceruloplasmin (IU/L)
40
0
Plasma Bilirubin (mg/dl)
4
2
0
Glutamate Oxalacetate Transaminase (S/F U/ml)
1200
600
0
Alkaline Phosphatase (SU/ml)
7.5
1.25
0 20 40
0 20 40 60 80 100
0 20 40 60 80
0 20 40 60 80 100 120
Time in days

Figure 2-2. Details of liver function tests and plasma copper in subacutely poisoned sheep and their controls. Sheep 133 and 116 were drenched with 1.5 g $CuSO_4 \cdot 5H_2O$/day as 1 percent solution during the periods shown. Dotted lines represent periods when hemolytic crisis was observed.

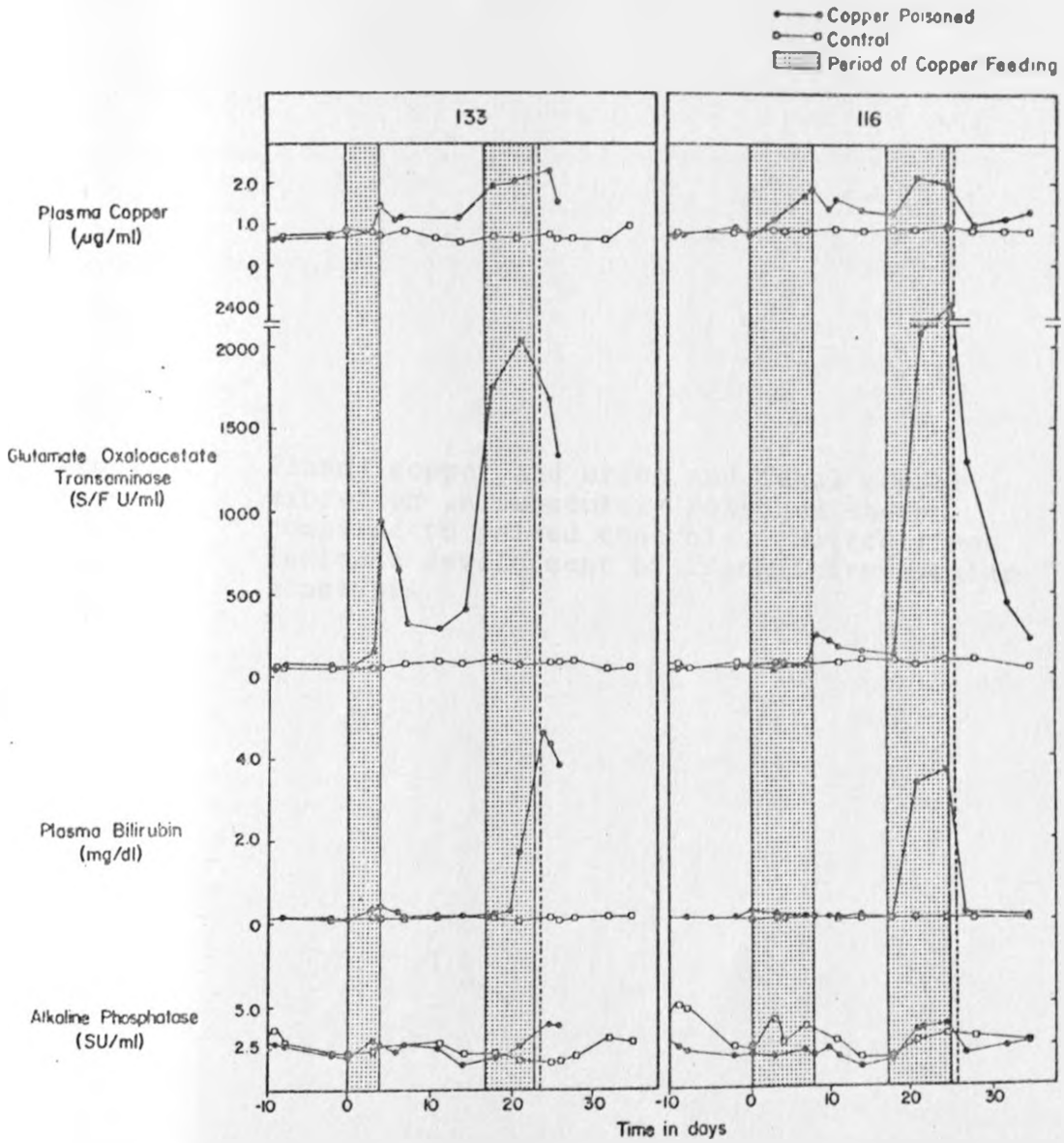

Copper Poisoned
Control
Period of Copper Feeding
133
116
Plasma Copper (μg/ml)
2.0
1.0
0
Glutamate Oxaloacetate Transaminase (S/F U/ml)
2400
2000
1500
1000
500
0
Plasma Bilirubin (mg/dl)
4.0
2.0
0
Alkaline Phosphatase (SU/ml)
5.0
2.5
-10
0
10
20
30
Time in days

Figure 2-3. Plasma copper and urine and fecal copper excretion in subacutely poisoned sheep compared to paired controls. Dotted lines indicate development of frank intravascular hemolysis.

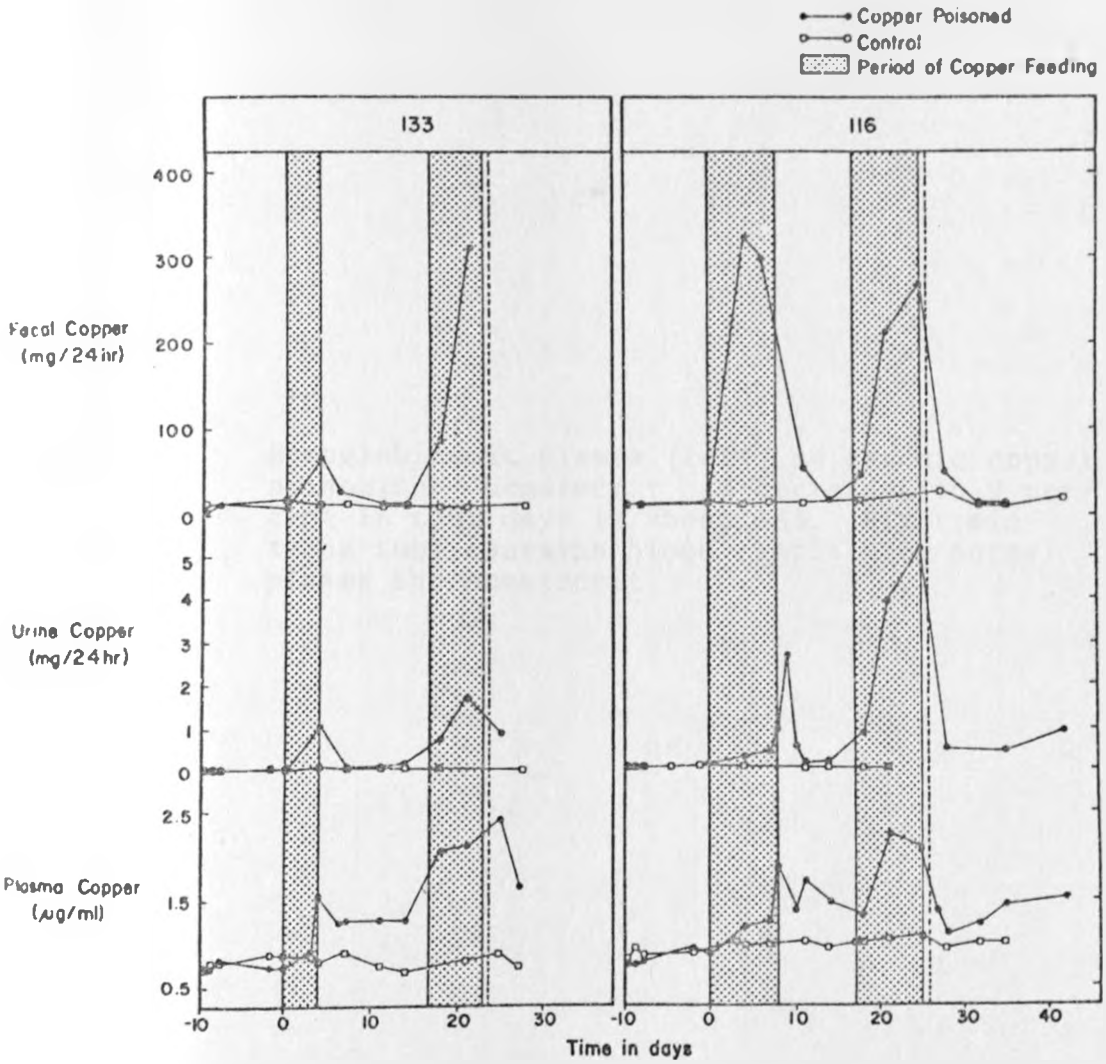

Copper Poisoned
Control
Period of Copper Feeding
133
116
Fecal Copper (mg/24 hr)
Urine Copper (mg/24 hr)
Plasma Copper (µg/ml)
400
300
200
100
0
5
4
3
2
1
0
2.5
1.5
0.5
-10
0
10
20
30
40
Time in days

Figure 2-4. Hemoglobinemic plasma (left) in chronic copper poisoning. Hematocrit had decreased to 9 percent in four days in sheep 133. Right Wintrobe tube contains blood sample with normal plasma and hematocrit.

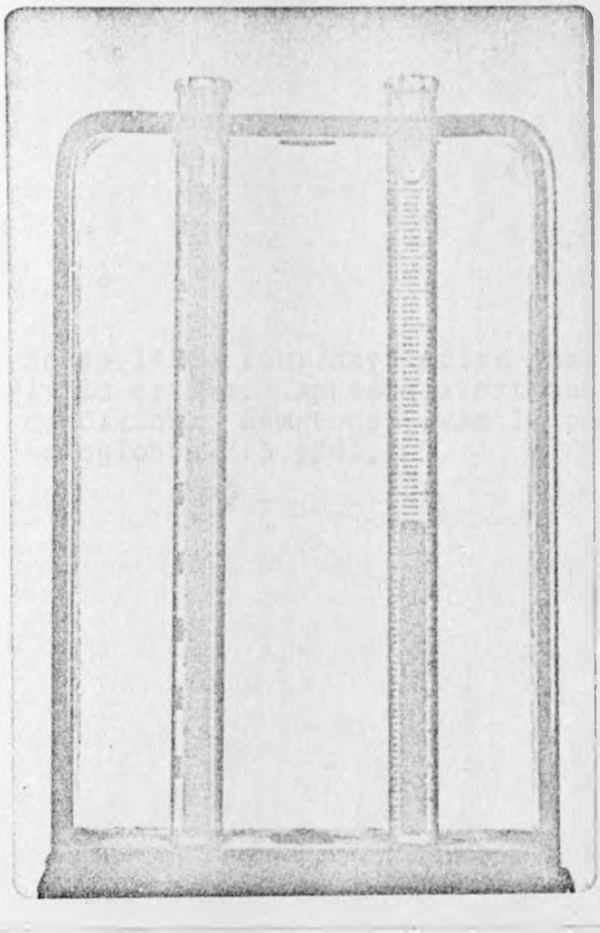


Figure 2-5. Sheep 1456, four days after onset of hemolytic crisis. Appears alert and in good bodily condition. Hematocrit was 12 per cent and hemoglobin 3.5 g/dl.

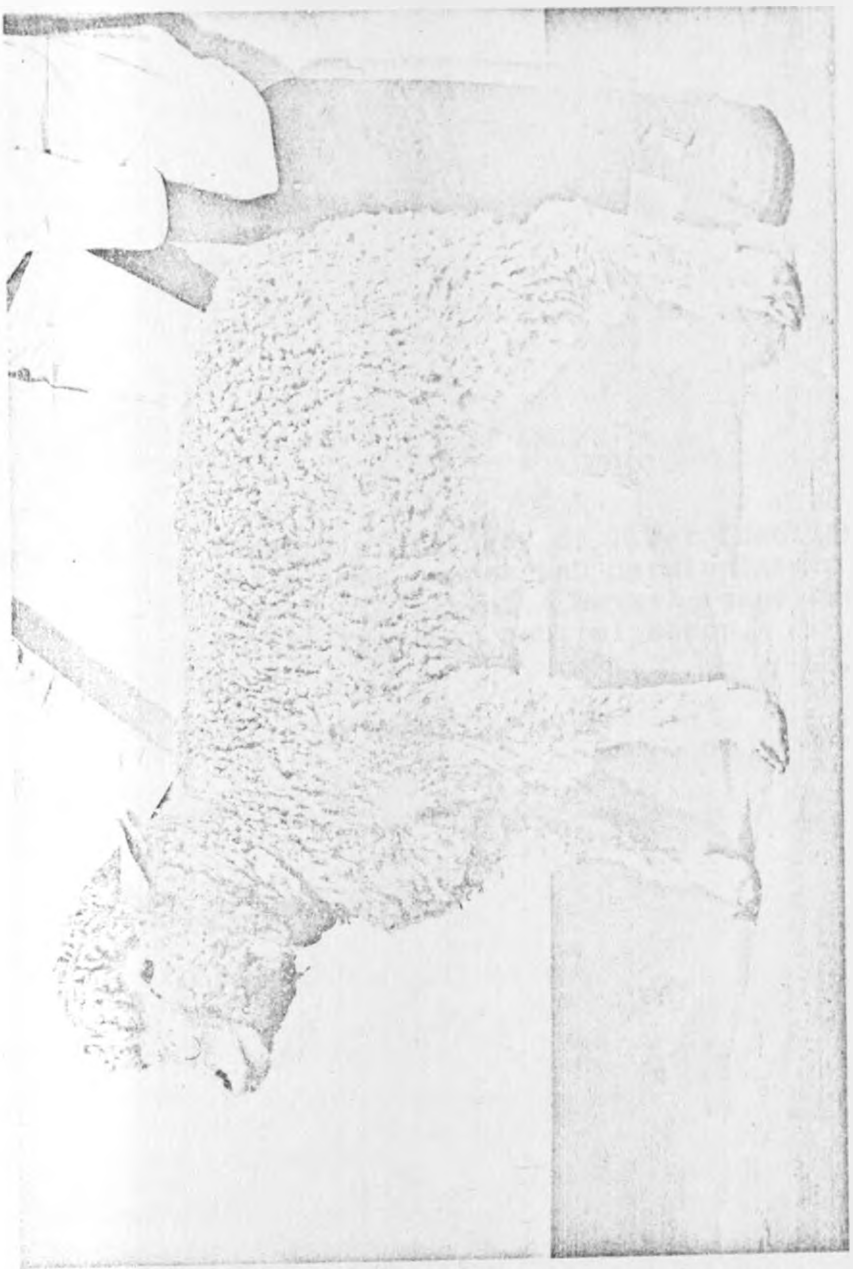


Figure 2-6. Summary of changes of liver function tests and plasma copper and ceruloplasmin in chronic copper experiment. Normal range indicates entire range in control sheep.

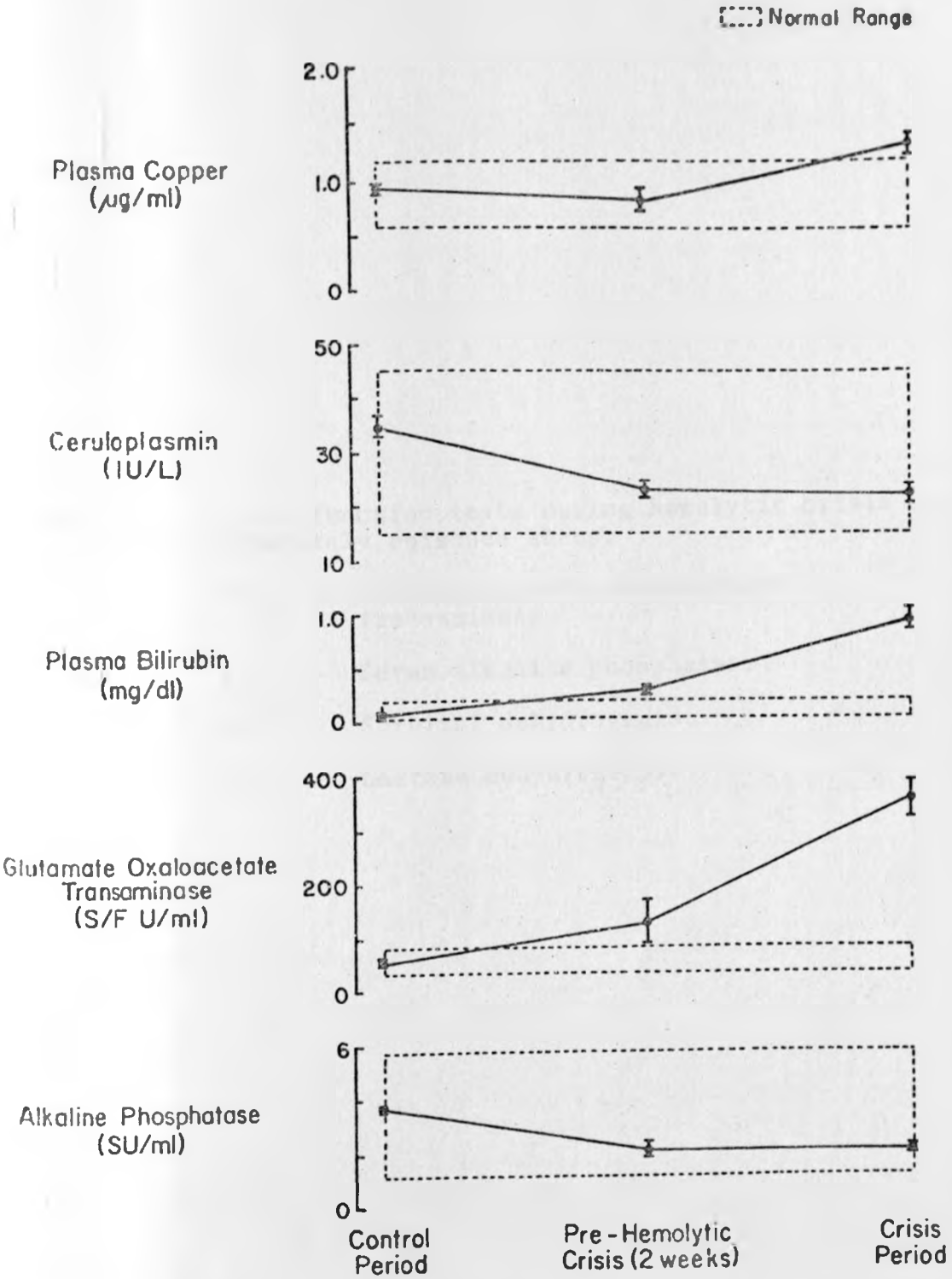

Normal Range
Plasma Copper
(µg/ml)
2.0
1.0
0
Ceruloplasmin
(IU/L)
50
30
10
Plasma Bilirubin
(mg/dl)
1.0
0
Glutamate Oxaloacetate
Transaminase
(S/F U/ml)
400
200
0
Alkaline Phosphatase
(SU/ml)
6
0
Control
Period
Pre-Hemolytic
Crisis (2 weeks)
Crisis
Period

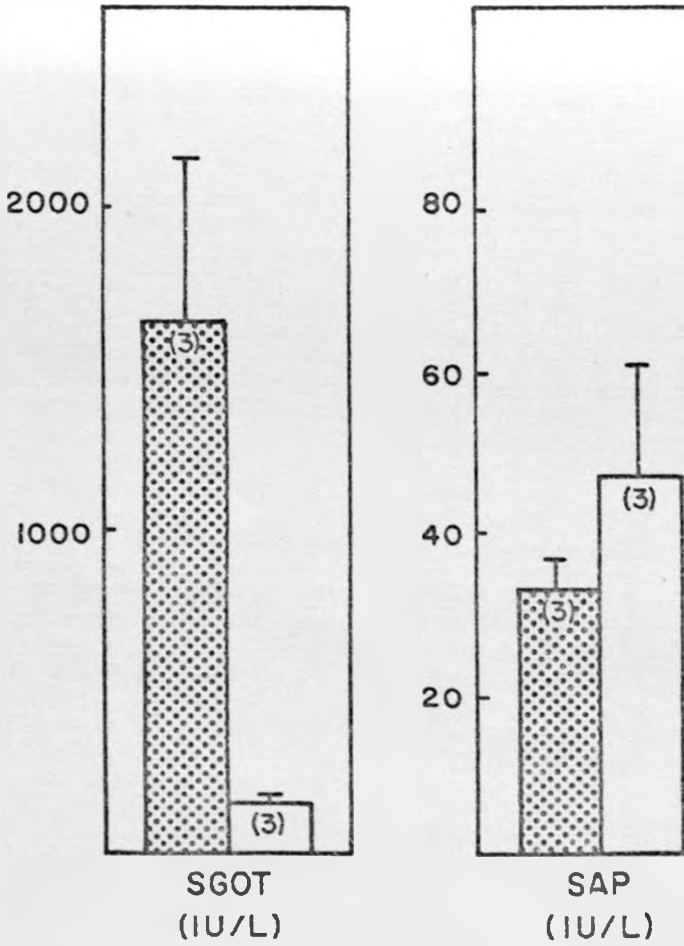
Figure 2-7. Liver function tests during hemolytic crisis in subacutely poisoned sheep.

SGOT - Serum glutamate oxaloacetate transaminase.

SAP - Serum alkaline phosphatase.

SDH - Sorbitol dehydrogenase.

LDH - Lactate dehydrogenase.


2000
1000
(3)
(3)
SGOT
(IU/L)
80
60
40
20
(3)
(3)
SAP
(IU/L)

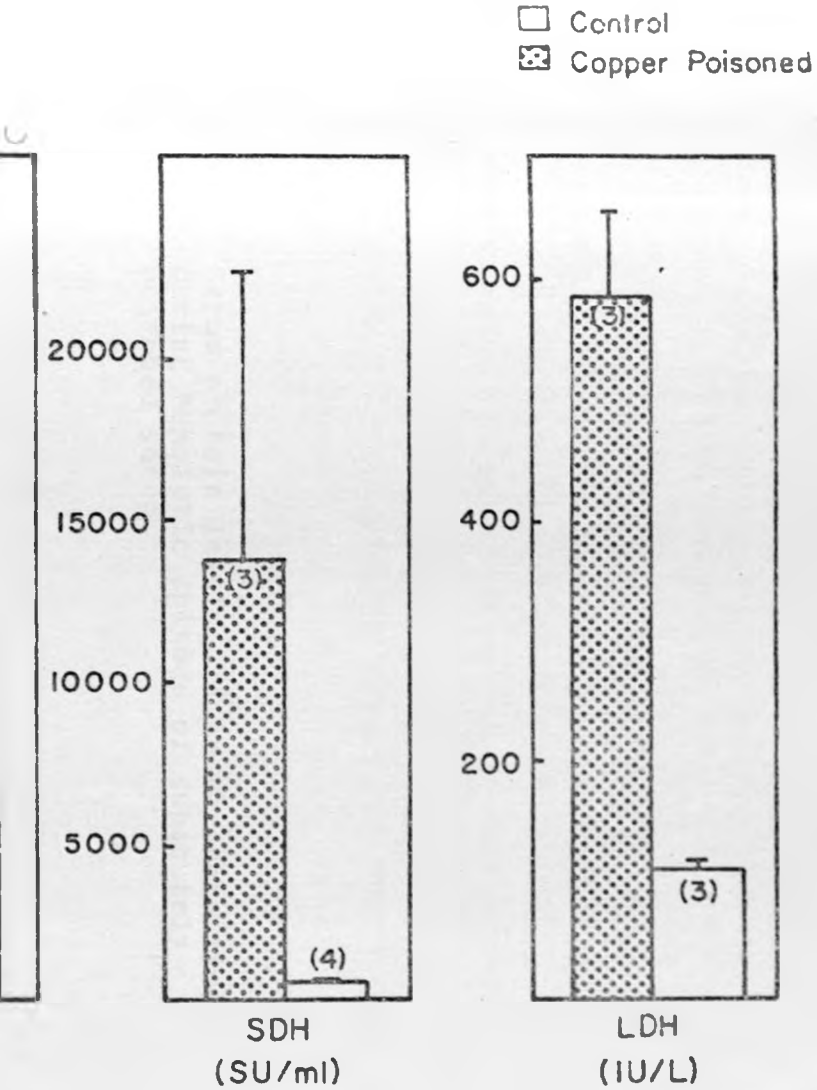
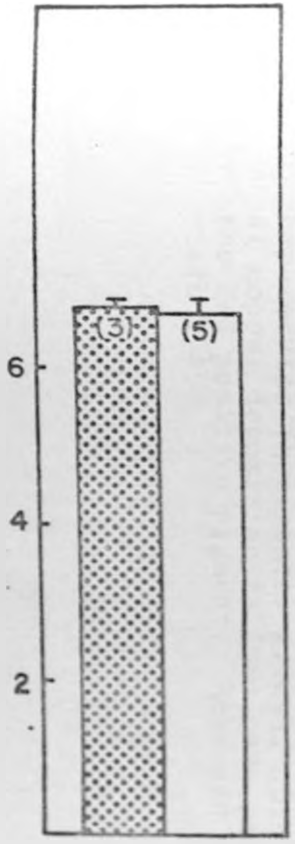
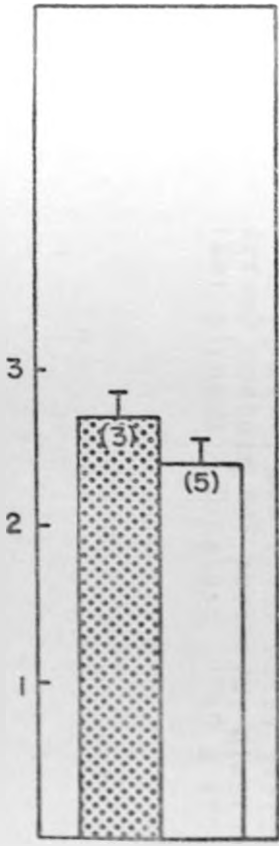

Control
Copper Poisoned
20000
15000
10000
5000
(3)
(4)
SDH
(SU/ml)
600
400
200
(3)
(3)
LDH
(IU/L)

Figure 2-8. Serum protein levels and prothrombin time during hemolytic episode of subacutely poisoned sheep.


6
4
2
(3)
(5)
Total Protein
(gm/dl)


3
2
1
(3)
(5)
Albumin
(gm/dl)

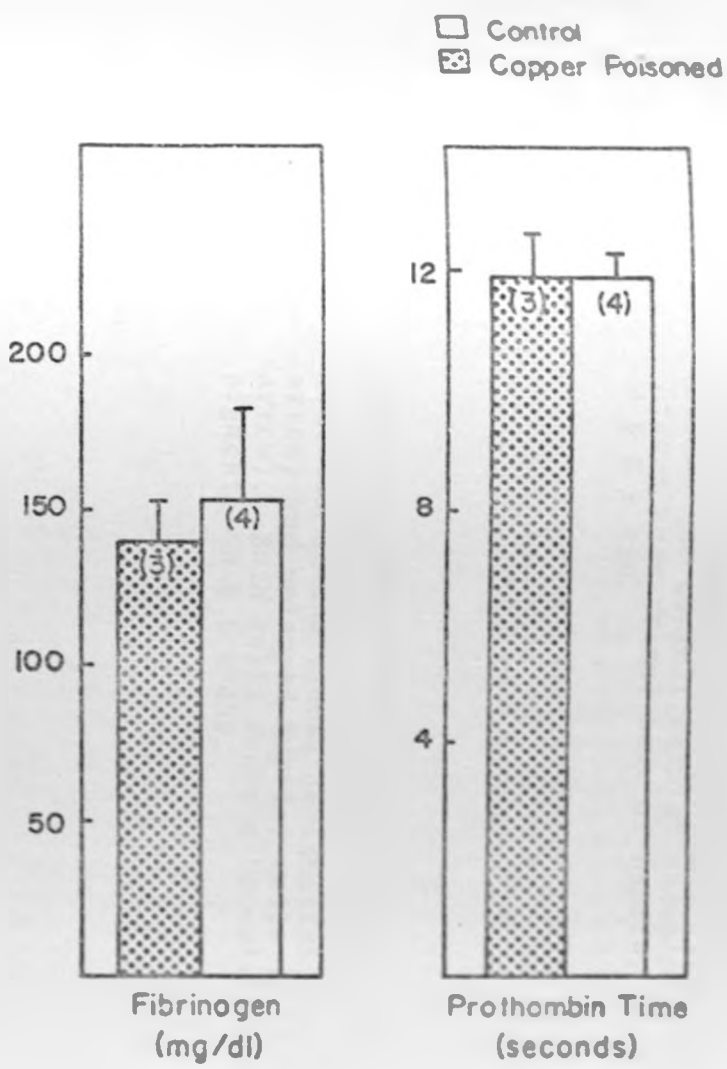

Control
Copper Poisoned
200
150
100
50
(3)
(4)
Fibrinogen
(mg/dl)
12
8
4
(3)
(4)
Prothombin Time
(seconds)

Figure 2-9a. Photomicrograph showing normal structure around the central vein of sheep's liver. Arrows show normal size of Kupffer cells. H & E x 450.

Figure 2-9b. Liver of sheep 1473. Killed on the first day of second hemolytic crisis. Swelling and necrosis of individual hepatocytes (horizontal arrow) and enlarged Kupffer cells (vertical arrow). Both cells contain brownish granular pigment. H & E x 450.

Figure 2-9c. Liver of sheep 1473. Kupffer cells (horizontal arrow) and liver cells (vertical arrow) contain PAS positive, diastase resistant pigment. Diastase PAS x 480.

Figure 2-9d. Liver of sheep 1473. Copper granules in the Kupffer cells (vertical arrow) and in the hepatocytes (horizontal arrow). Distribution of copper deposition follows same pattern as the PAS positive pigment. Rubeanic acid stain x 450.

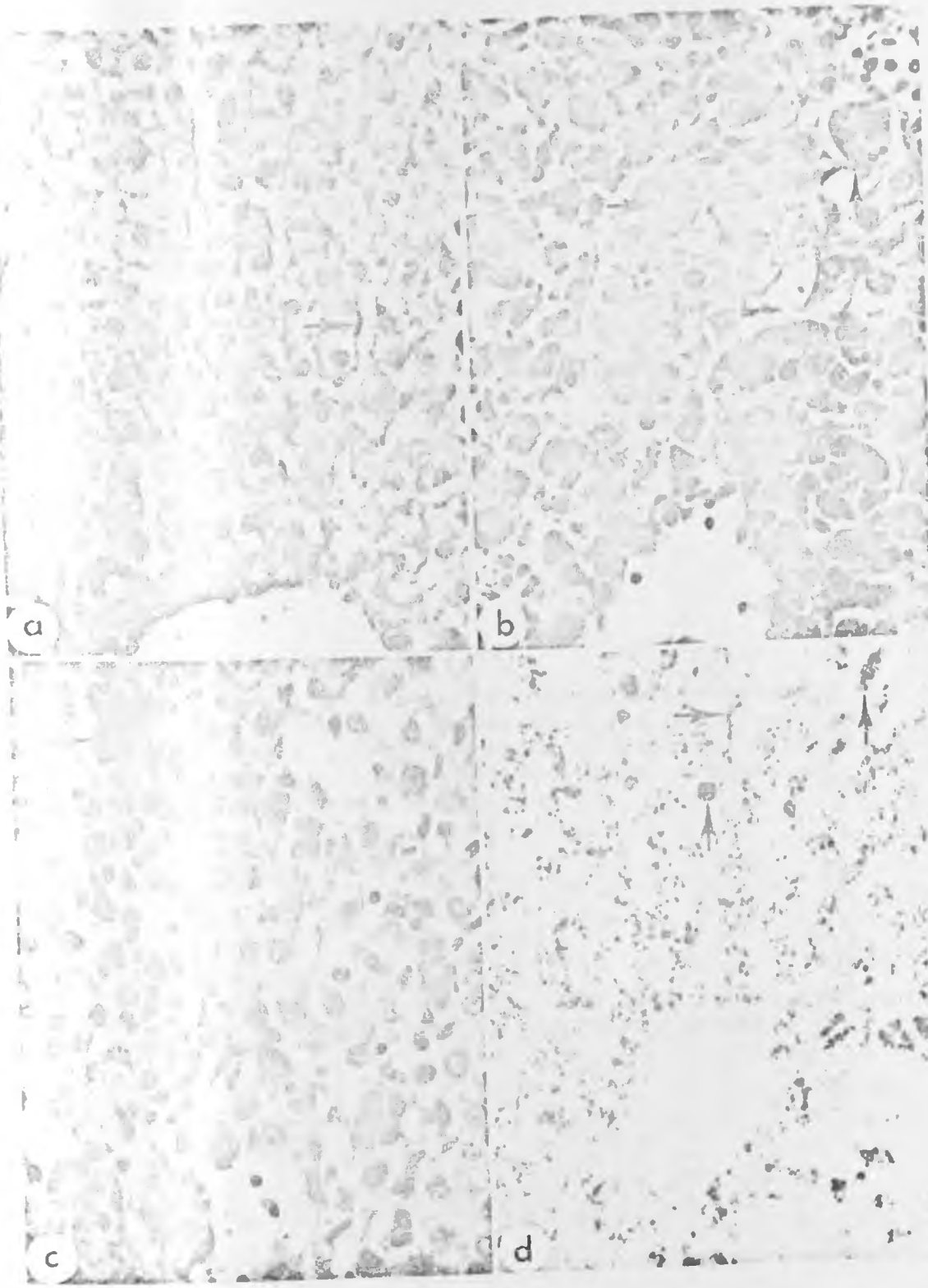
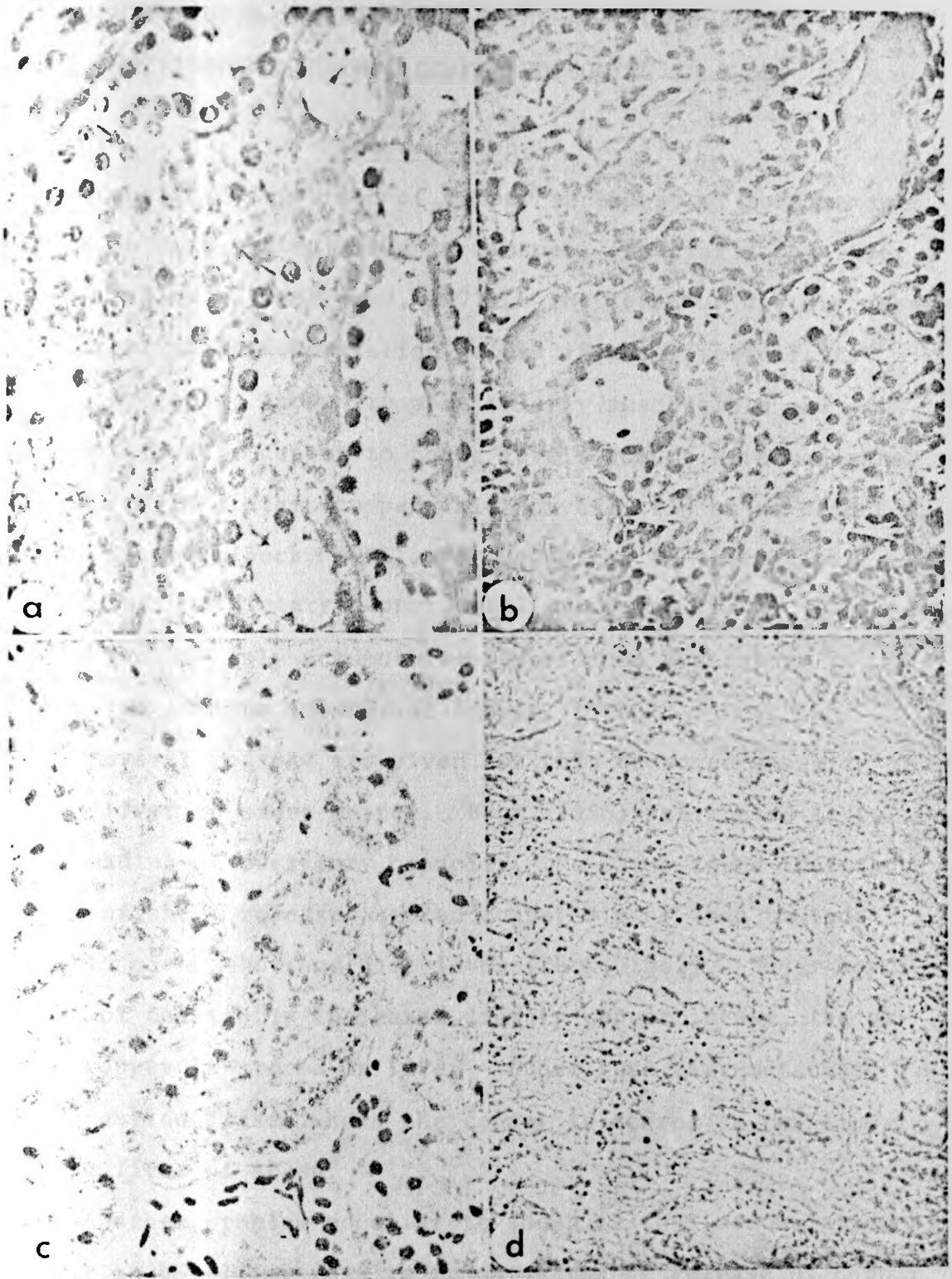

a
b
c
d

Figure 2-10a. Kidney of sheep 371. Killed 4 days after appearance of hemolysis. Proximal tubular necrosis with loss of basement membrane (upper left arrow). Lumen of the tubule filled with necrotic debris (central arrow). H & E x 480.

Figure 2-10b. Kidney of sheep 133 (subacute copper poisoning) showing hemoglobin casts in the proximal tubules. Casts were also present in the glomerulus. BUN in this sheep reached 170 mg/dl before euthanasia. H & E x 250.

Figure 2-10c. Kidney of sheep 1473, chronically poisoned with copper. Killed on first day of second crisis. The epithelium of proximal tubules contains iron positive pigment. Prussian Blue stain x 450.

Figure 2-10d. Kidney of sheep 1473. Proximal tubular cells also contain copper granules. Rubeanic acid stain x 450.


a
b
c
d

CHAPTER III

SUBCELLULAR DISTRIBUTION OF HEPATIC COPPER IN EXPERIMENTAL CHRONIC COPPER POISONING IN SHEEP

Introduction

Continued ingestion of copper in excess of requirements leads to some accumulation in tissues, especially the liver, of all animals (Underwood, 1977). The level of dietary copper intake at which accumulation occurs in the liver differs among species. Sheep are particularly susceptible and accumulation of copper to toxic levels occurs with moderate increases in dietary copper which have little or no effect on other species (Beck, 1955). Accumulation in sheep liver occurs with a dietary copper level of 20 ppm (Beck, 1955) while the rat only accumulates copper when fed copper in excess of 100 ppm (Milne and Weswig, 1968).

Several reasons are given for this enhanced capacity of sheep liver to store copper. Beck (1955) attributed it to overloading of excretory capacity. He later found that the liver of sheep released stored copper slower than that of rabbits given the same dose level (Beck, 1962). Under conditions of constantly increased dietary copper intake, the excess copper is stored in liver. Under such circumstances, the question arises where the copper is stored so tenaciously in the liver of sheep.

Another problem peculiar to sheep is that accumulation of copper in the liver results in catastrophic release of copper

causing an acute hemolytic crisis (Todd, 1969). Factors which permit or instigate the release of copper are poorly defined but are usually regarded as being stressful situations such as a falling plane of nutrition, excessive exercise or a sudden check to food intake (Jubb and Kennedy, 1970). Whatever the precipitating cause, it appears that excess copper in the liver is stored in a labile compartment from which it is readily released.. This compartment has not been positively identified.

The purpose of this study was to characterize the subcellular distribution of copper in chronically poisoned sheep. It was hoped that such information would serve as the basis for further investigations of the metabolic defect in ovine copper metabolism which makes this species uniquely susceptible to clinical copper poisoning.

Materials and Methods

Details on sheep and treatments were given in the second chapter of this thesis. Within five minutes after a sheep was euthanized, the liver was removed and a portion of approximately 100 g was placed in an iced solution of 0.25 M sucrose and transported to the laboratory.

Subcellular Fractionation

All tissue homogenation procedures were completed in a cold room maintained between 1C and 4C. The liver fractionation procedure followed for chronically poisoned sheep was that of Gregoriadis and Sourkes (1967) with minor modifications.

The suspending medium consisted of 0.25 M sucrose - 10 mM HEPES solution buffered to pH 7.5 with potassium hydroxide.

A piece of the liver was weighed, minced with scissors and suspended in 4 volumes of 0.25 M sucrose - 10 mM HEPES solution. The tissue in homogenization medium was transferred to a 50 ml glass homogenizer.[a] Three teflon pestles with clearances of 0.066, 0.030 and 0.020 cm were used in that sequence to grind the tissue, one minute of continuous homogenization for each. The weight and volume of the homogenate (H_1) were recorded.

An aliquot of H_1 was reserved for copper and protein analysis and the rest centrifuged[b] at 600 xg for 10 minutes at 4C. The pellet which contained nuclei and other cell debris was suspended in 40 ml sucrose-HEPES solution, re-homogenized and re-centrifuged as before. The supernatants were combined (S_1) and volume recorded. The 600 g pellet (P_1) was suspended in 30 ml sucrose-HEPES solution and stored at -20C. A portion of S_1 was reserved and the rest centrifuged[b] at 14,000 xg for 30 minutes. The 14,000 g pellet was suspended in 20 ml sucrose-HEPES solution and re-centrifuged to yield crude mitochondria (P_2). The combined supernatants (S_2) were centrifuged at 105,000 xg for 120 minutes in an ultracentrifuge.[c] This yielded the microsomal pellet (P_3) while the

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[a] Potter-Elvehjem tissue grinder, A. H. Thomas, Philadelphia, Pa. 19135.

[b] Model J21b, Beckman Instruments Inc., Fullerton, Cal. 92631.

[c] Model L5-50, Beckman Instruments Inc., Fullerton, Cal. 92631.

supernatant (S_3) represented the cytosol.

P_2 was suspended in 10 ml sucrose-HEPES solution while P_3 was suspended in 5 ml of the solution. All the volumes and weights of the supernatants and the pellets were recorded. The fractions were stored at -20C until copper and protein analyses were performed.

In the subacute experiment, the homogenate was prepared using 4 volumes of sucrose-HEPES and the cytosol prepared as described above except that centrifugation lasted only 60 minutes.

Separation of Metal Binding Proteins

A piece of the liver stored in ice-cold sucrose-HEPES solution was blotted dry and weighed. It was homogenized in 2.5 volumes of 1 mM sodium bicarbonate solution. The homogenate (H) was centrifuged[a] at 105,000 xg for 120 minutes. The supernatant (cytosol) was either used immediately for gel chromatography or stored at -20C.

When it was time for Sephadex column separation, frozen samples of cytosol were thawed at room temperature, thoroughly mixed and centrifuged at 14,000 xg RPM for 30 minutes.[b] Five ml of supernatant were applied to a column of Sephadex G-100 (Pharmacia, Uppsala, Sweden) through a 0.45 mu millipore

[a] Model L5-50, Beckman Instruments Inc., Fullerton, Cal. 92631.

[b] Model 25, Beckman Instruments Inc., Fullerton, Cal. 92631.

filter unit.[a] The glass column employed in each case was 2.5 cm x 87 cm and elution was done with tris-buffer at pH 7.4 to which was added 0.01 percent beta-mercaptoethanol. Approximately 200 3 ml fractions were collected.

To enable molecular weight calculation of the cytosolic proteins, the column was calibrated with bovine serum albumin (MW 68,000), chymotrypsin (MW 25,700), ovalbumin (MW 45,000) and cytochrome C (MW 12,400). Void volume was taken as that point at which the first protein peak appeared. Molecular weights of the cytosolic proteins were calculated from regression of known molecular weights on the ratio (Ve/Vo) of elution volume (Ve) to void volume (Vo) (Fig. 3-1).

Determination of Copper

The subcellular fractions were thawed at room temperature and thoroughly mixed. Measured volumes were placed in tared porcelain crucibles and their weights recorded. In the chronic copper experiment the fractions analyzed were P_1, P_2, P_3, S_3 and H_1, while in the subacute copper experiment only the S_3 and homogenates were analyzed.

The samples were freeze dried for 24 hours.[b] They were transferred to a drying oven at 100C for up to 3 hours. Dry weight was recorded and the samples placed in a muffle furnace

[a] Millipore filter, Millipore Corp, Bedford, Mass. 01730.

[b] Model 10-800, Virtis freeze drier, Virtis Co., Gardiner, N.Y. 12525.

and ashed at 500C overnight. The ash was dissolved in 5 ml 4N HCl and the solution transferred to 25 ml volumetric flasks and diluted to volume with deionized distilled water. The ash from P_3 was dissolved in two ml acid, transferred to 10 ml flasks and diluted to volume. All samples were done in duplicate.

Duplicate aliquots of whole liver and kidney were processed for copper determination as described above. Copper analysis was done by aspirating the dissolved ash into an atomic absorption spectrophotometer.[a] The instrument was calibrated with copper standards made from analytical grade $CuSO_4 \cdot 5H_2O$. Zinc concentrations were measured in the livers and kidney using the same atomic absorption spectophotometer.

Fractions from the Sephadex column were analyzed for copper by direct aspiration into the atomic absorption spectrophotomer.

Protein Determination

Protein concentration was measured using the method of Lowry et al, (1951). Subcellular fractions from the sucrose-HEPES homogenate were analyzed manually and optical density (OD) read at 500 nm.[b] Concentration was calculated from a standard curve made with bovine serum albumin standards run simultaneously with the unknown samples.

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[a] Model 305B Perkin-Elmer, Norwalk, Conn. 06856.

[b] Model 25, Beckman Instruments Inc., Fullerton, Cal. 92631.

Protein in the fractions from the Sephadex column were analyzed using an automated modification of the Lowry protein analysis.[a] Bovine serum albumin also was used as the reference protein

Acid phosphatase of the liver homogenates and subcellular fractions was analyzed using Sigma methodology.[b] In the reaction, p-nitrophenyl phosphate hydrolysis, catalyzed by acid phosphatase, yields p-nitrophenol which forms a yellow color with alkali. To 0.5 ml of 0.4 percent, p-nitrophenyl phosphate (substrate) was added 0.5 ml acetate buffer, pH 4.85 and 0.2 ml of sample. In the blank tube 0.2 ml water was substituted for the sample. Tubes were incubated for 30 minutes at 37C and color developed by adding 5 ml 0.1 N sodium hydroxide (NaOH). Optical density (OD) was read at 400 nm.[c] Activity was calculated from acid phosphatase standard curve and expressed in sigma units per ml (SU/ml). Since samples had been diluted 1 in 10 or 1 in 100, the activity was adjusted accordingly. Final acid phosphatase activity of the fraction was calculated per gram of fresh liver.

Electron Microscopy

Percutaneous liver biopsies for electron microscopy (EM) were obtained from each sheep when it had a hemolytic

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[a] Autoanalyzer I, Technicon Corp., Tarrytown, N.Y. 10591.

[b] Sigma Technical Bulletin No. 104, Sigma Chemical Co., St. Louis, Mo. 63178.

[c] Model 25, Beckman Instruments Inc., Fullerton, Cal. 92631.

crisis. Control sheep were also biopsied at the same time.

Biopsy site was located with the sheep in a standing position. A point was taken at the 10th intercostal space on an imaginary line drawn from the tuber coxae to the point of the elbow on the right side of the animal. After routine surgical preparation of this area, the biopsy was taken by directing the needle cranio-ventrally at 30 to 45^{o} along the imaginary line.

The biopsy was immediately placed in 4 percent glutaraldehyde in phosphate buffer pH 7.2. Another biopsy was fixed in 10 percent buffered formalin for light microscopy. The EM biopsy was post-fixed in 2 percent osmium tetroxide in Millorigs' buffer for one hour, stained in 2 percent uranyl acetate for 45 minutes, dehydrated in ethanol and then embedded in Epon-Araldite. Sections were mounted on carbonized Formvar-coated grids, stained with lead citrate and examined in an electron microscope.[a]

Results

Liver and Kidney Copper and Zinc

Liver and kidney copper and zinc concentrations are shown in Table 3-1. The liver copper concentration of the control sheep varied between 329 and 888 ppm on a dry matter (DM) basis (674 ± 93.7) while that of the copper poisoned sheep ranged from 2456 to 4108 ppm DM (3126 ± 287). Mean hepatic

[a] Model 301, Phillips Electron Microscope.

copper concentration of the copper poisoned sheep was statistically higher than that of the control sheep ($p < 0.05$).

Zinc concentrations in the livers of copper poisoned animals, 167 ± 21.9 (110 - 255) ppm DM were not significantly different from that of the control sheep, 151 ± 24.9 (81 - 216) ppm DM.

The mean control kidney copper levels of 5 sheep was 25.9 ± 10.7 ppm. The mean kidney copper concentration of poisoned sheep was 239 ± 260 ppm, with an observed range between 35.2 and 671 ppm. Although the mean values differed by a factor of 10, this was not statistically significant. Three of the chronically poisoned sheep (1450, 1456, 371) had kidney copper levels within the range of control sheep while the other three sheep (1473 poisoned chronically, and 116 and 133 from the subacute poisoning study) had remarkably elevated kidney copper levels.

Kidney zinc concentration in poisoned sheep (158 ± 14.2 ppm DM) was not significantly different from that of the control sheep (138.2 ± 26.4).

Electron Microscopic (EM) Examination

EM examination of the livers containing large amounts of copper exhibited irregularly shaped dense granular bodies enclosed by a single membrane. These dense bodies were located primarily near the bile canaliculi of the hepatocytes (Fig. 3-2) and in the enlarged Kupffer cells (Fig. 3-3). The distribution of the granules in electron micrographs was

similar to the copper distribution observed under light microscopy. It is believed that these dense bodies are lysosomes containing an increased amount of copper. Lysosomes observed in the liver from the control sheep were fewer and generally were stained more lightly (Fig. 3-4).

Fractionation Studies

All the metal concentrations are expressed as ug/g fresh tissue. The subcellular copper distribution in chronically poisoned sheep is shown in Table 3-2 and Figures 3-5 and 3-6.

In the control sheep, approximately 40 percent of liver copper was present in the 14,000 g fraction (P_2) while the 600 g (P_1) and cytosol (S_3) fractions each contained approximately 20 percent of the hepatic copper. Less than 10 percent of the liver copper was present in the 105,000 g pellet (P_3). In the copper poisoned sheep, 44 percent of liver copper was in 600 g fraction while the 14,000 g pellet and cytosol accounted for 23.9 percent and 16.2 percent, respectively.

The distribution data show that there was increased copper storage primarily in the 600 g pellet. The mean copper concentration in this 600 g fraction was 310 $\pm$ 32 ug/gm fresh liver which was statistically higher ($p < 0.01$) than that of the control sheep (43.6 $\pm$ 5.0 ug/g fresh liver). The cytosol fraction of copper poisoned sheep stored 123.3 $\pm$ 30.4 ug copper/g fresh tissue compared to 39.9 $\pm$ 6.6 ug copper/g fresh liver in the cytosol fraction of liver from control

sheep. The difference was significant ($p < 0.05$). The increases in copper storage observed in the 14,000 g pellet (157 ± 48.6 vs 71.5 ± 9.5 ug/g fresh liver) and in the 105,000 g pellet (33.5 ± 10.5 vs 17.3 ± 9.4) were not significant.

Copper dosing increased the concentration of copper in the livers of subacutely poisoned sheep. The two poisoned sheep had liver copper concentrations of 632 and 567 mg/g fresh tissue while the controls had 167 and 191 mg copper/g fresh liver. The liver cytosol of subacutely poisoned sheep stored 40 percent of the copper compared to 10 to 12 percent in the control sheep (Table 3-3).

Acid Phosphatase Distribution

Acid phosphatase distribution in subcellular fractions is shown in Figure 3-7. The highest proportion of acid phosphatase (44.4 ± 4.78 percent) was recovered in the 600 g pellet of the liver of copper fed sheep. The 14,000 g pellet yielded 14.3 ± 5.5 percent and the cytosol, 17.9 ± 0.03 percent. Acid phosphatase activities in control sheep were similar except that there was higher activity in the cytosol. Distribution of acid phosphatase was very similar to that of copper (Fig. 3-7).

Copper Distribution in the Cytosol

Cytosolic proteins were separated on columns of Sephadex G-100. The distribution of protein and copper is shown in Figures 3-8, 3-9, 3-10 and 3-11. Previous calibration of the

Sephadex G-100 column with proteins of known molecular weight indicated that the protein peaks corresponding to the copper Peaks I, II and III represented protein fractions of molecular weights of approximately 75,000, 35,000 and 12,000 respectively.

Copper distribution in control sheep 1451 and 1461 was similar. The pattern for sheep 1451 is shown in Figure 3-8. Most cytosolic copper was associated with the high molecular weight fraction (Peak I). Peak II had less copper and there was almost none in Peak III. Control sheep 1454 (Fig. 3-9) with hepatic copper concentration of 777 ppm DM had an increased amount of copper in Peak III.

Three of the chonically poisoned sheep showed a copper distribution similar to that of sheep 1473 (Fig. 3-10). Notable here is the increased amount of copper in both Fractions I and III. Cytosolic copper distribution in sheep 1450 (Fig. 3-11) showed an increased proportion of copper in Peak I but Peak III did not show the typically high copper concentration present in other sheep. The total cytosolic copper concentration in 1450 was lower than all other chronically poisoned sheep.

All the copper poisoned sheep had a fourth copper peak (fraction IV) which was associated with a protein or peptide with a molecular weight in the range 3,000 to 5,000. This peak had the highest copper binding in subacutely poisoned sheep (Fig. 3-12).

The significance of Fraction III is indicated by calculating the concentration of copper as a function of the protein

content (Fig. 3-13). The proteins in the low molecular weight region are significantly enriched with copper in copper-loaded sheep. There was also a significant correlation ($r = 0.84$) between the peak specific activity of Fraction III (ug copper/mg protein) and the hepatic copper concentration on a dry matter basis ($p < 0.05$) (Fig. 3-14).

Discussion

The liver copper concentrations recorded in the copper poisoned sheep ranged from 2456 to 4108 ppm DM. Similar values have been reported previously by several authors in experimental chronic copper poisoning (Gopinath et al., 1974; Ishmael et al., 1971; Gopinath and Howell, 1975). The mean liver copper concentration in the control sheep was 674 ± 93.7 ppm DM. This was higher than the reported normal of 100-400 ppm copper (Underwood, 1977). Some of the controls had copper levels higher than the 800 ppm safe level observed by Jubb and Kennedy (1970) but did not show any clinical or biochemical evidence of toxicity. Todd (1969) observed that when sheep were kept indoors for extended periods, chronic copper poisoning was one of the possible hazards. Several reasons were given for this increased susceptibility of housed sheep to excessive copper accumulation. These could include copper supplementation in excess of requirement, but the most likely cause was the imbalance between copper and molybdenum found in concentrates (Todd, 1972).

The kidney copper concentrations in the copper poisoned

sheep showed a wide variation (35.2 - 671 ppm DM). The mean 239 ± 260 ppm DM did not differ significantly from that of the control sheep (25.9 ± 10.7) because of the wide variation in the individual poisoned sheep.

Elevated kidney copper level is reported to be diagnostic for chronic copper poisoning since it is only increased after hemolytic episodes when blood copper concentration is very high (Gopinath et al., 1974; Eden, 1940). The two sheep which had low kidney copper levels (1450 and 1456) underwent only mild hemolytic crises from which they were recovering when they were killed. The kidney copper levels might only be temporarily elevated.

There was no significant difference between hepatic zinc concentration of the copper poisoned and control sheep (Table 3-1). This contradicts the findings of Philip (1973) who found decreased liver zinc concentration in the sheep which had a high liver copper level and which suffered hemolytic jaundice. Copper-zinc antagonism is well documented and results from similarity in their chemical properties (Hill and Matrone, 1970). These two metals compete for binding sites on metallothionein (Evans et al., 1970a). This competition can occur at the absorption sites (Starcher, 1969; Van Campen and Scaife, 1967) which restricts zinc uptake or in the liver where high concentration of one metal may result in a redistribution of the other metal either in the subcellular organelles (Philip, 1973) or among cytosolic binding proteins (Bremner and Marshall, 1974a,b). In the latter case, hepatic zinc

concentration may be normal as observed in the present work. Displacement from the sites would only render the metal unavailable for metabolic functions.

In the study reported here hemolytic crisis was accompanied by increased presence of intracytoplasmic granules along the bile canaliculi in the hepatocytes and in Kupffer cells (See Chapter II of thesis). These were PAS positive and diastase resistant. They stained negatively for iron and positively for copper with rubeanic acid. Electron microscopic studies confirmed that these densely stained granules were contained in lysosomes in the liver of copper poisoned sheep but were less dense in control livers.

Several authors have found that in the livers of animals given excess copper, the element appears to be stored in the lysosomes (Verity et al., 1967; Worwood and Taylor, 1969; McNatt et al., 1971). In rabbits given copper EDTA intraperitoneally, the liver parenchymal cells took up the copper in a finely dispersed form but it was transferred into lysosomes which appeared to be produced in large numbers after the first injection of copper (Wessel et al., 1966). Early stages of Wilson's disease have the diffuse copper distribution but in the advanced disease, copper is found exclusively in the lysosomes (Goldfischer and Sternlieb, 1968). Sequestration of copper in the lysosomes supposedly protects the cell from toxic effects of copper (Goldfischer et al., 1970) but could also be deleterious when lysosomal membranes are damaged through peroxidation which releases acid hydrolyses (Lindquist,

1968).

The fractionation studies demonstrated that in control sheep with hepatic copper concentration ranging from 329 ppm to 888 ppm DM, 37 percent of the hepatic copper was stored in the 14,000 g pellet. The 600 g and supernatant (cytosol) fractions stored about 20 percent each. In the copper loaded sheep, liver sampled at post-mortem showed significant apparent accumulation in the 600 g pellet (44 percent).

In the normal sheep, the location of most copper in the 14,000 g pellet agrees with the findings of Philip (1973). Most other reports incriminate the liver cytosol as the main storage site in normal animals of other species (Porter et al., 1961; Thiers and Vallee, 1957; Gregoriadis and Sourkes, 1967). When hepatic copper concentrations are higher than normal, copper is stored predominantly in the crude mitochondrial fraction in all animals (Hermann and Kun, 1961). Similar mitochondrial storage to that found in sheep is observed in the livers of immature rats (Gregoriadis and Sourkes, 1967) and immature bovines (Porter et al., 1961).

Several authors (Hermann and Kun, 1961; Gregoriadis and Sourkes, 1967; Milne and Weswig, 1968; Feldman et al., 1972; Verity et al., 1967) have assigned a predominant role to the crude mitochondrial fraction in retention of excess copper. Gregoriadis and Sourkes (1967), Lal and Sourkes (1971) and Philip (1973) described a changing pattern of copper storage in which the mitochondrial fraction showed early accumulation

but at high hepatic copper concentration, the nuclear fraction continued to accumulate while the mitochondrial storage plateaued. Philip (1973) observed copper accumulation in the cytosol only at the highest hepatic copper attained in her work which was 465 ug/g fresh liver or 1395 ppm DM. Usually, copper poisoned sheep can accumulate more copper as demonstrated in the present work where copper levels ranged between 2456 and 4108 ppm DM. Based on the copper concentration, there was increased copper storage in all the fractions but this increase was only statistically significant in the 600 g and cytosol fractions (Table 3-2). However significant increase in storage relative to the amount of protein occurred only in the 600 g pellet.

Distribution of acid phosphatase, which is predominantly lysosomal, demonstrated that nearly 45 percent was recovered from the 600 g pellet in the livers of copper poisoned sheep. Previous histologic and electron microscopic reports indicated that during hemolytic crisis, there was marked copper accumulation in the lysosomes. Normally, these organelles sediment with the 14,000 g pellet. The high recovery of acid phosphatase in the 600 g pellet indicates that a greater amount of lysosomes are trapped in this fraction or sediment with intact cells. Salwant et al. (1964) emphasized the difficulty in isolating lysosomes during differential centrifugation due to their particulate properties. When these organelles are loaded with metal as observed in the electron micrographs, the increased density will force them to

sediment in the 600 g pellet as recorded in the present work.

The liver cytosol of the two subacutely poisoned sheep accumulated up to 40 percent of liver copper. This observation was made previously by Milne and Weswig (1968). It is possible, based on the present findings, that when copper is rapidly accumulated in the liver, the cytosol fraction may play a significant role in storage of the excess metal.

Sephadex separation of cytosol proteins demonstrated that copper was associated with three main protein peaks in the sheep with normal and elevated hepatic copper concentrations. These peaks were designated as I (MW 75,000), II (MW 35,000) and III (MW 12,000). They have previously been demonstrated by Bremner and Marshall (1974a,b) and Philip (1973). In livers of copper poisoned sheep, the second protein peak was associated with a diffuse copper plateau. Fraction II was characterized by Bremner and Marshall (1974b) as superoxide dismutase as it showed the dismutase properties described by McCord and Fridovich (1969). Fraction III was characterized by Bremner and Marshall (1974b) as metallothionein.

The observation that in livers with low copper concentration, most copper was bound in Fraction I supports the findings of Philip (1973). Hepatic copper accumulation was accompanied by increased binding of copper in metallothionein (Fraction III) and an increase in Fraction I but no observable increase in Fraction II which also agrees with observations of Philip (1973). The significance of Fraction III as copper

binding site was demonstrated by calculating the concentration of copper as a function of the protein content. There was little difference from controls in the copper to protein ratio for protein of high and medium molecular weights, but the proteins in Fraction III were significantly enriched with copper in copper poisoned sheep. Similar observations were made by Bloomer and Sourkes (1973) who concluded that the copper binding protein was constitutive. Electropherograms of the proteins in the metallothionein peak from normal and copper loaded livers were identical. Bloomer and Sourkes (1973) considered this as evidence that copper loading did not induce synthesis of a protein that was not already in the liver. The copper to protein ratio varied with hepatic copper concentration as it did in the present work.

Following the finding by Bremner and Davies (1974) that copper injected into rats induced appearance of a copper binding fraction by active protein synthesis, Riordan and Gower (1975) recorded the presence of copper binding proteins which differed from metallothionein in their low cystine content. Copper loading increased the concentration of those proteins. On the contrary, Premakumar et al., (1975b) isolated a protein which they named copper-chelatin from livers of copper injected rats. This protein was absent in livers of control rats. The protein was different from cadmium or zinc induced metallothionein (Winge et al., 1975).

No copper-chelatin has been isolated from livers of ruminants. Bremner and Marshall (1974a,b) observed that

copper accumulation in Fraction III was dependent on zinc being sufficient. The total concentration of zinc and copper bound by the fraction was a direct function of liver zinc. Copper was not bound by the fraction if zinc was deficient. They isolated a protein which was characterized as metallothionein. Zinc was necessary for synthesis or stabilization of the protein. Bremner (1976) has recognized the same relationship in the liver of pigs.

Based on the observation that copper to protein ratio in the copper loaded sheep varied with total hepatic copper and not the protein content of the cytosol, it seems reasonable to conclude that the binding protein content remained the same as copper accumulated. Saturation of the copper binding protein occurs at high copper concentration resulting in the presence of free unbound copper which causes observed liver injury (Ishmael et al., 1971). It would be interesting to isolate the protein and determine the copper binding constants.

A fourth copper peak appeared in copper loaded livers. The significance of this fraction was not determined but would probably be due to binding of copper by low molecular weight peptides when Fraction III is saturated.

Copper accumulation in Fraction I was suggested by Bremner and Marshall (1974a) to result from non-specific binding of copper by high molecular weight proteins. The copper to protein ratio remained constant in this fraction as liver copper increased except in cases of very high copper concentration, for example, the ratio increased abruptly in the liver of

1473 (Fig. 3-13).

Summary

Copper supplementation increased storage of the metal in the liver of sheep. Kidney copper concentrations were elevated above control levels in the sheep that went through severe hemolytic crises. Two sheep which reacted mildly had nearly normal copper levels. The latter indicated that the little amount of copper that might have been trapped in the kidney was rapidly cleared when hemolysis ceased.

Increased copper storage did not alter zinc concentration in the livers and kidneys of copper poisoned sheep.

Subcellular studies demonstrated that there was increased copper concentration in all the fractions at the time of hemolytic crisis. Most copper was recovered from the 600 g pellet (approximately 50 percent), and nearly a similar recovery of acid phosphatase was made from this fraction. The histologic and electron microscopic studies showed marked deposition of copper in the lysosomes during this period. It is therefore concluded that the lysosomal granules, especially those with increased density due to presence of copper, precipitated with 600 g pellet giving the observed high copper concentration.

Sephadex separation of cytosol gave three main copper peaks associated with proteins of molecular weights 75,000, 35,000 and 12,000. In the control sheep most copper was bound to protein Peak I (MW 75,000). Some control sheep with liver copper above 700 ppm DM showed increased binding of copper

in Fraction III (MW 12,000). In copper poisoned sheep, a large copper peak appeared in the third fraction.

The third fraction (MW 12,000) revealed an increased copper to protein ratio which was highly correlated with total hepatic copper concentration. This was interpreted as meaning that the amount of copper binding protein in the fraction did not change much but it continually bound copper. Saturation of this fraction might result in free copper which is injurious to the cells.

The significance of the fourth fraction is not evident. It may be free copper being taken up by low molecular weight peptides. This fraction may be associated with appearance of signs as it was highest in the subacutely poisoned sheep which suffered very severe hemolytic crises.

Table 3-1. Copper and Zinc Concentrations in Liver and Kidney of Control and Copper Poisoned Sheep, Dry Matter Basis.

	Copper (ppm)		Zinc (ppm)	
Control Sheep	Liver	Kidney	Liver	Kidney
1451	888	28	115	95
1454	777	66.6	150	123
1461	329	11.6	81	100
114	677	16.1	216	240
129	706	7.4	195	133
Mean ± SEM	674 ± 93.7	25.9 ± 10.7	151 ± 24.9	138.2 ± 26.4
Copper Poisoned Sheep				
1450	2804	49.8	110	118
1456	2873	35.2	142	112
371	3917	65.8	139	169
1473	4108	440.0	150	181
116	2601	671.0	255	194
133	2456	173.0	208	178
Mean ± SEM	3126 ± 287	239 ± 260	167 ± 21.9	158 ± 14.2
Significance (Student's t test)	$p < 0.05$	ns	ns	ns

Table 3-2. Subcellular Hepatic Copper Distribution in Chronic Copper Feeding Experiment (ug Copper/g Wet Tissue).

Control Sheep	Fraction					Percent Recovery
	H_1	P_1	P_2	P_3	S_3	
1451	196	34	84.5	10	51.8	91
1454	203	50.8	53	36	39.2	88
1461	179	46	77	6	28.8	88.4
Mean ±SEM	192 ± 7.1	43.6 ± 5.0	71.5 ± 9.5	17.3 ± 9.4	39.9 ± 6.6	89.1 ± 0.94
Copper Poisoned Sheep						
1450	576	250	203	29	64	94
1456	596	273	83	13	104	79
371	864	394	272	63	117	97
1473	776	326	70	29	208	81
Mean ±SEM	703 ± 70	310 ± 32	157 ± 48.6	33.5 ± 10.5	123.3 ± 30.4	87.8 ± 4.5
Significance (Student's t test)	$p < 0.01$	$p < 0.01$	ns	ns	$p < 0.05$	ns

No. 373 died unexpectedly and tissue not included because of autolysis.

Table 3-3. Copper Concentration (ug/g Fresh Tissue) in Liver Homogenate and Cytosol of Subacutely Poisoned Sheep and Their Controls.

	Liver Fraction	
Control Sheep	Homogenate	Cytosol
114	167	16
129	191	24
Copper Poisoned Sheep		
116	632	196
133	567	228

Figure 3-1. Correlation between molecular weight and ratio of elution volume to void volume in Shephadex G-100 column chromotography.

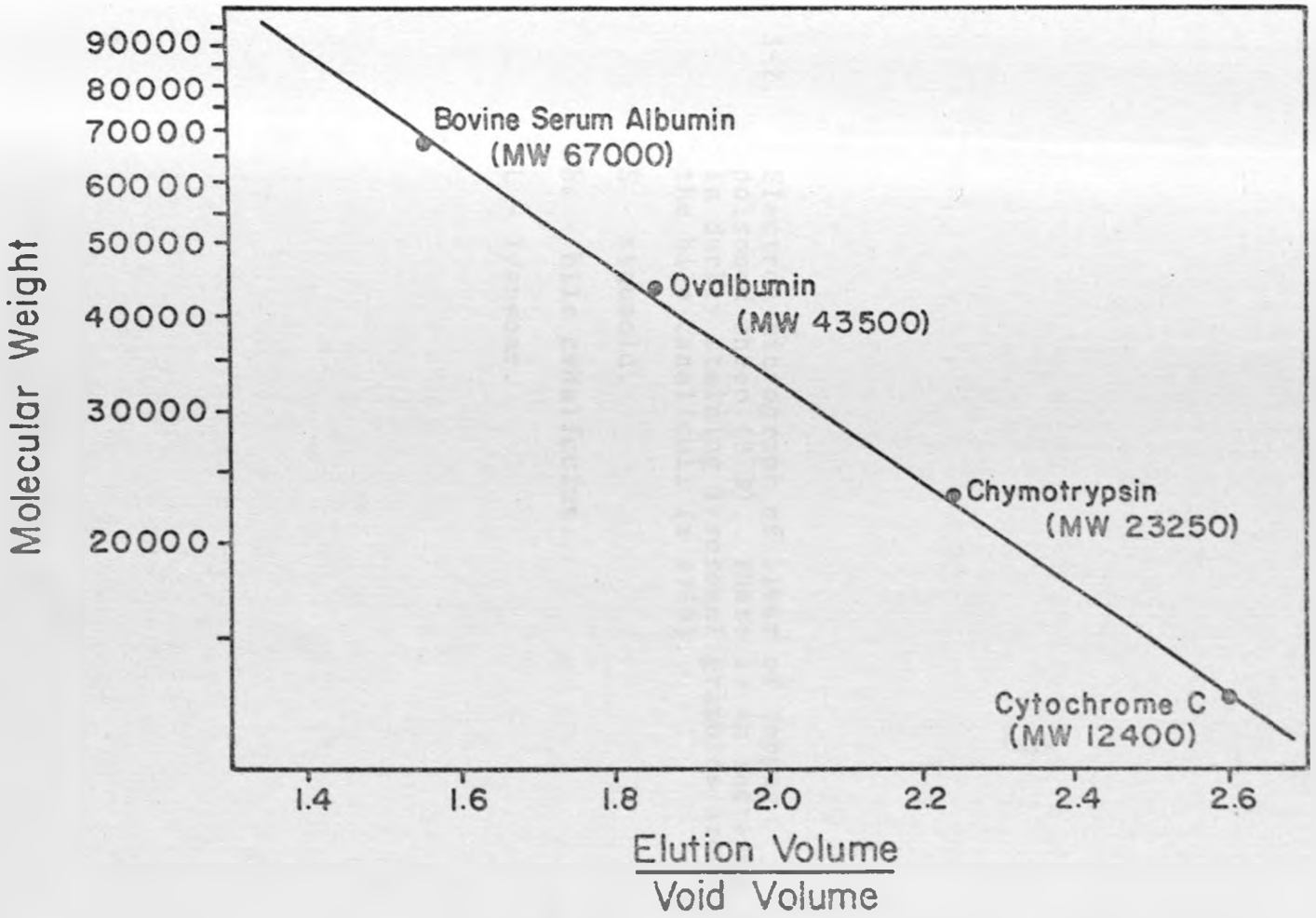

Molecular Weight
90000
80000
70000
60000
50000
40000
30000
20000
Bovine Serum Albumin
(MW 67000)
Ovalbumin
(MW 43500)
Chymotrypsin
(MW 23250)
Cytochrome C
(MW 12400)
1.4
1.6
1.8
2.0
2.2
2.4
2.6
Elution Volume
Void Volume

Figure 3-2. Electron micrograph of liver of copper poisoned sheep (373). There is an increase in darkly staining lysosomal granules around the bile canaliculi (x 4750).

S - sinusoid.

bc - bile canaliculus.

L - lysosome.

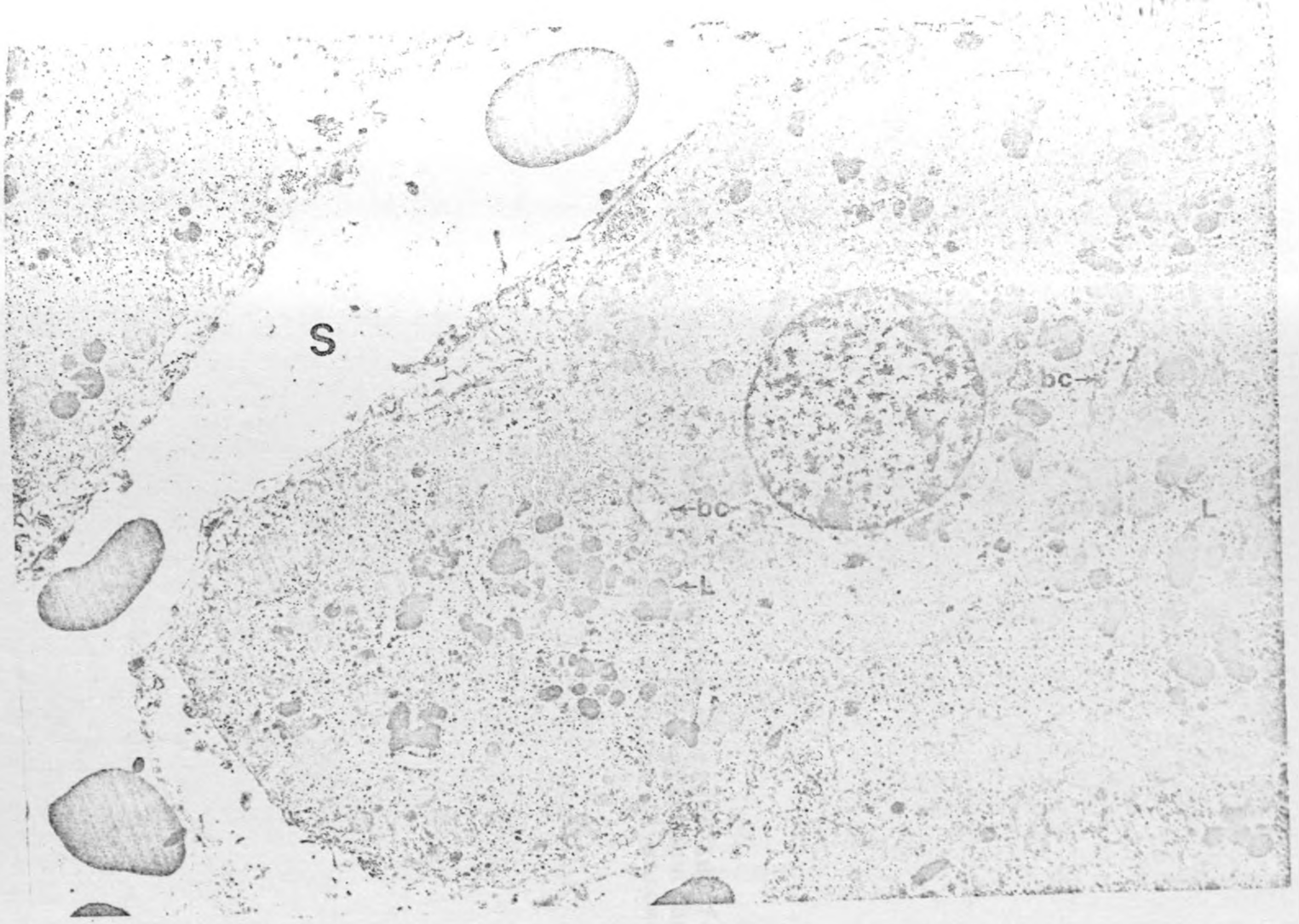

S
bc
bc
L
L

Figure 3-3. Electron micrograph showing enlarged Kupffer cell which is filled with dark granules contained within membranous vacuoles (lysosomes) (x 6250).

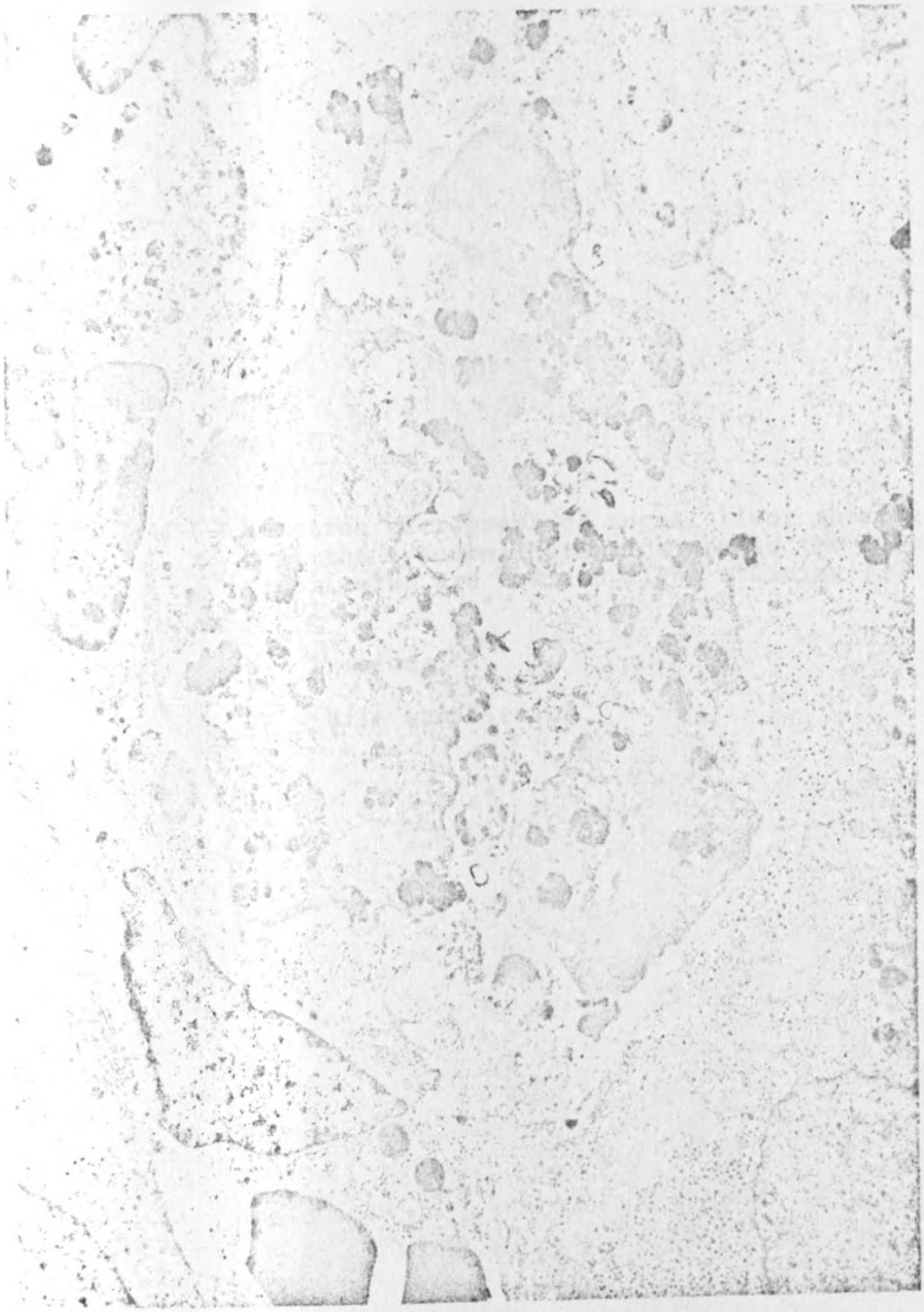


Figure 3-4. Electron micrograph of normal liver showing that the lysosomal granules around the bile canaliculus are few and light staining (x 8500).

L - lysosome.

bc - bile canaliculus.

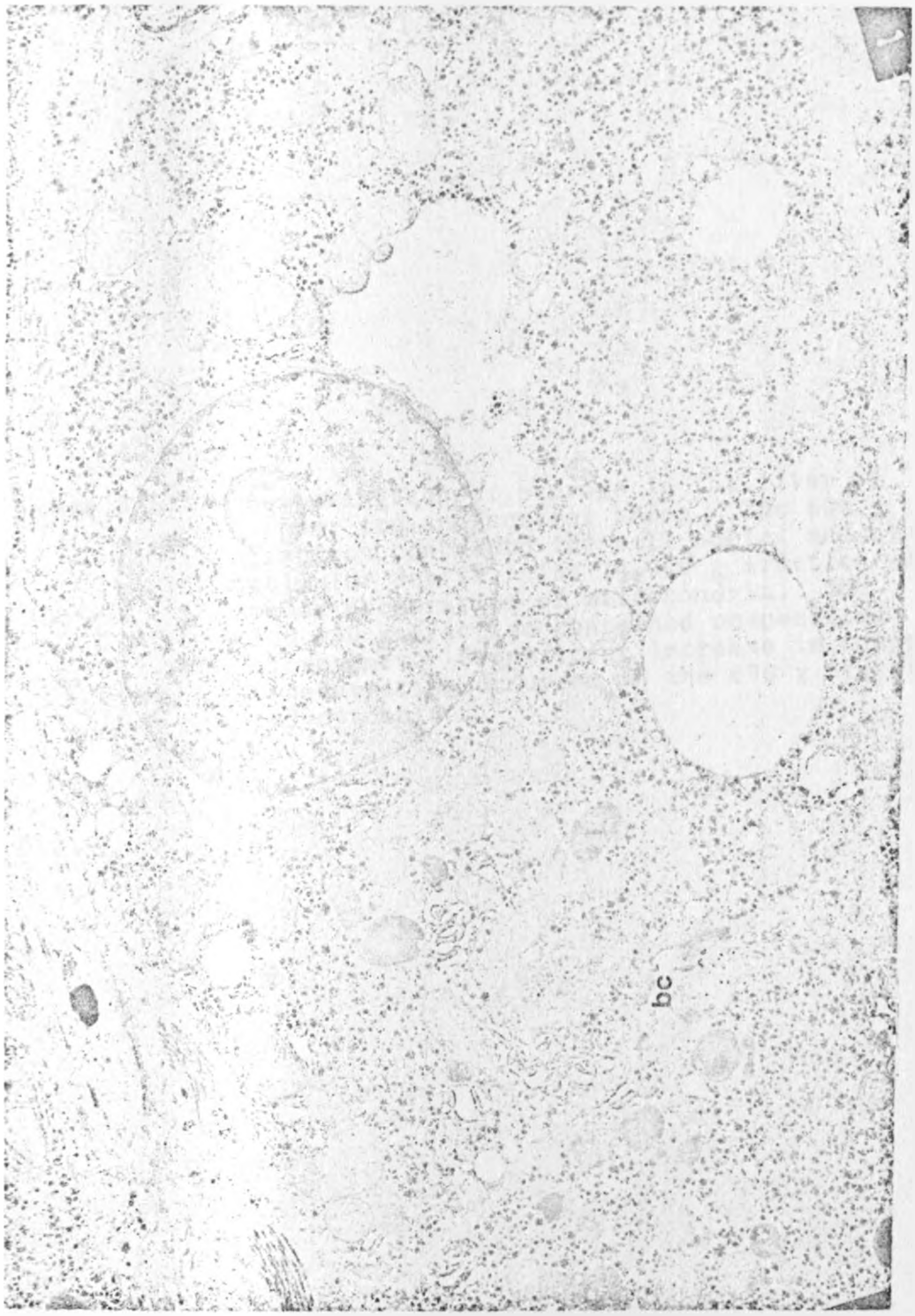

bc

Figure 3-5. Comparing copper storage in the liver of copper fed and control sheep. The 600 g fraction contained the cell nuclei and other cellular debris. The 14,000 g fraction was a crude preparation of mitochondria. The 105,000 g fraction contained unspecified microsomes. Significant increase in copper concentration occurred in the 600 g fraction and cytosol.

Liver Copper (µg/gm fresh tissue)
700
600
500
400
300
200
100
Homogenate
(p < 0.01)

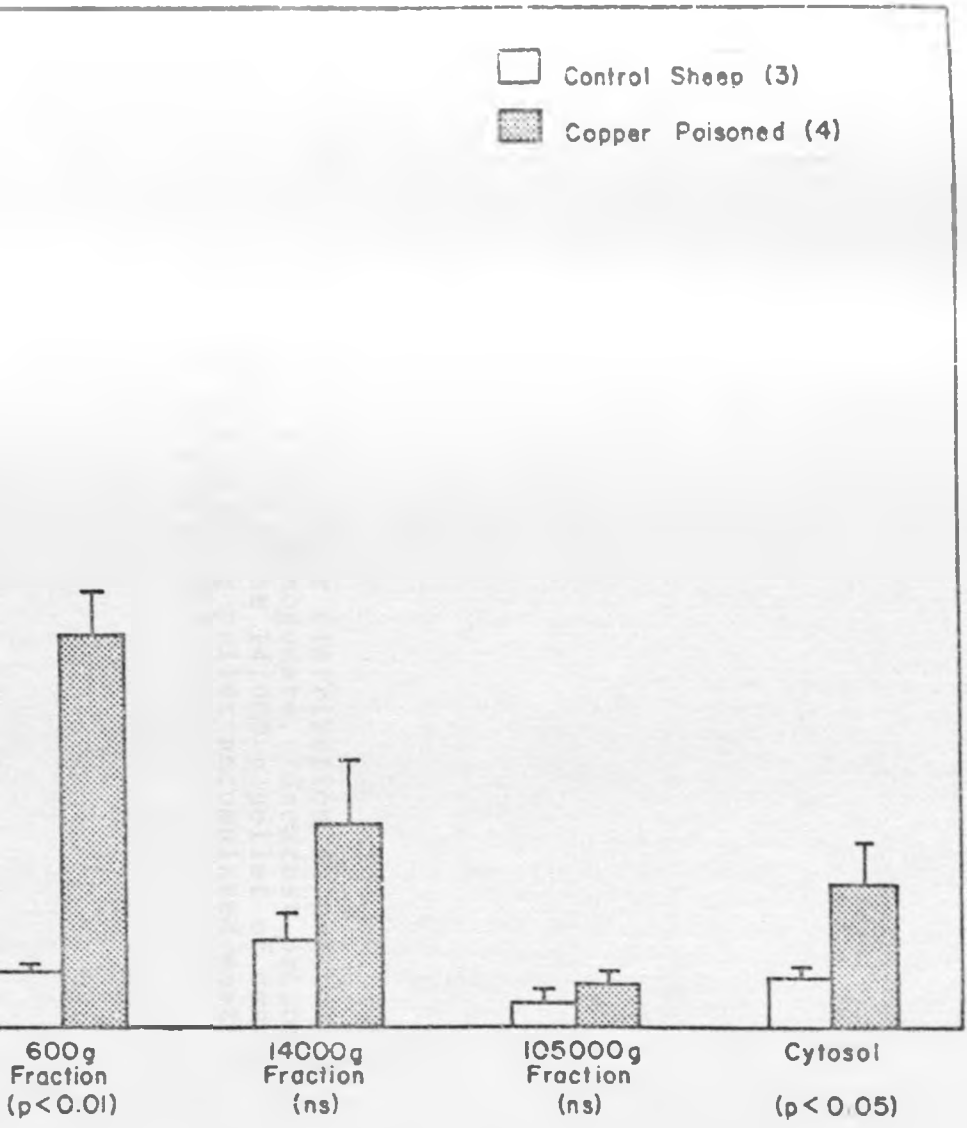

Control Sheep (3)
Copper Poisoned (4)
600g Fraction (p<0.01)
14000g Fraction (ns)
105000g Fraction (ns)
Cytosol (p<0.05)

Figure 3-6. Subcellular copper distribution as percent of original liver homogenate. Greatest proportion of copper is in the 14,000 g pellet of control livers. The 600 g pellet accumulated most copper in copper fed sheep.

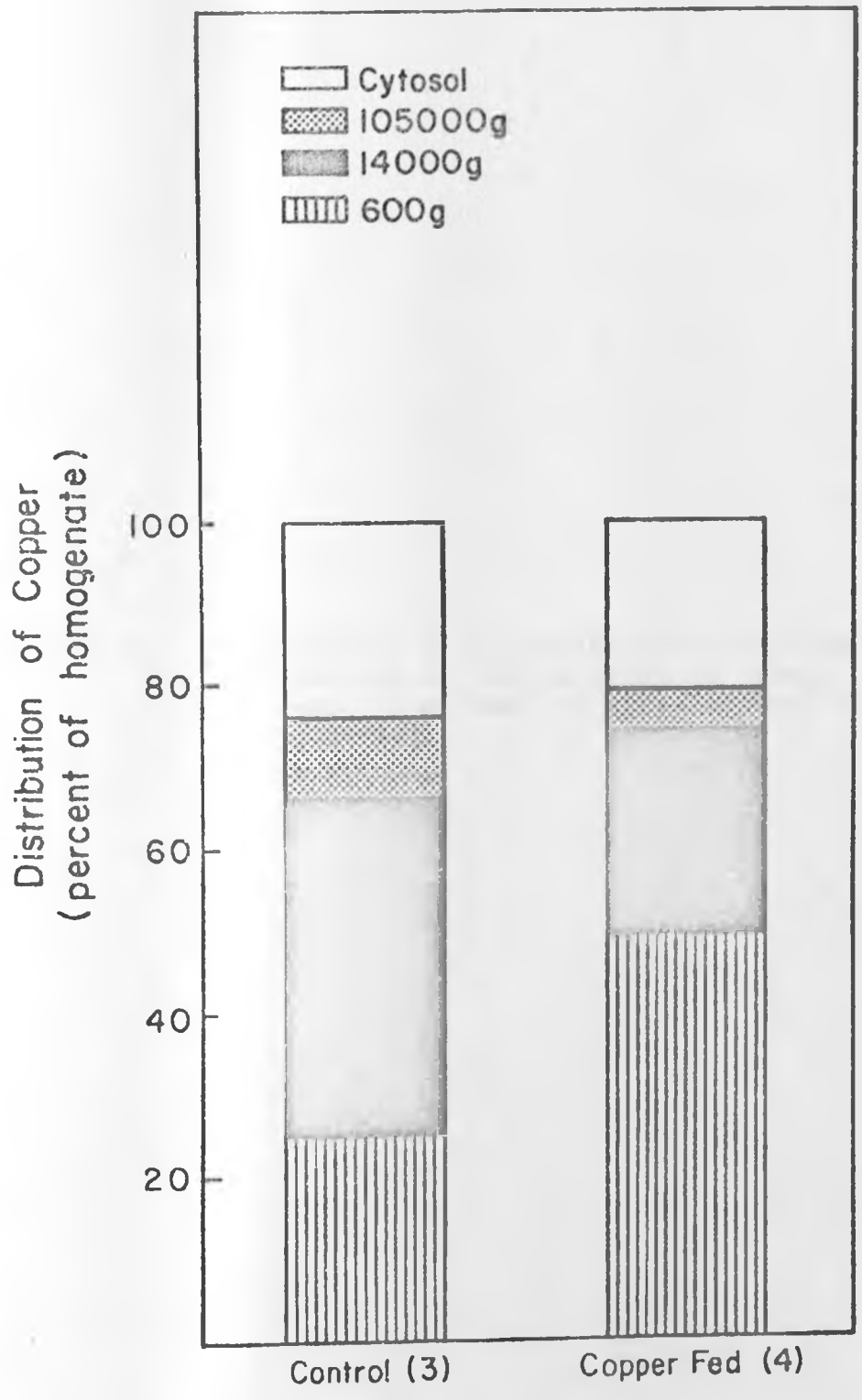

Cytosol
105000g
14000g
600g
Distribution of Copper
(percent of homogenate)
100
80
60
40
20
Control (3)
Copper Fed (4)

Figure 3-7. Subcellular distribution of copper and acid phosphatase in copper poisoned sheep. There is about 50 percent recovery of each in the 600 g pellet.

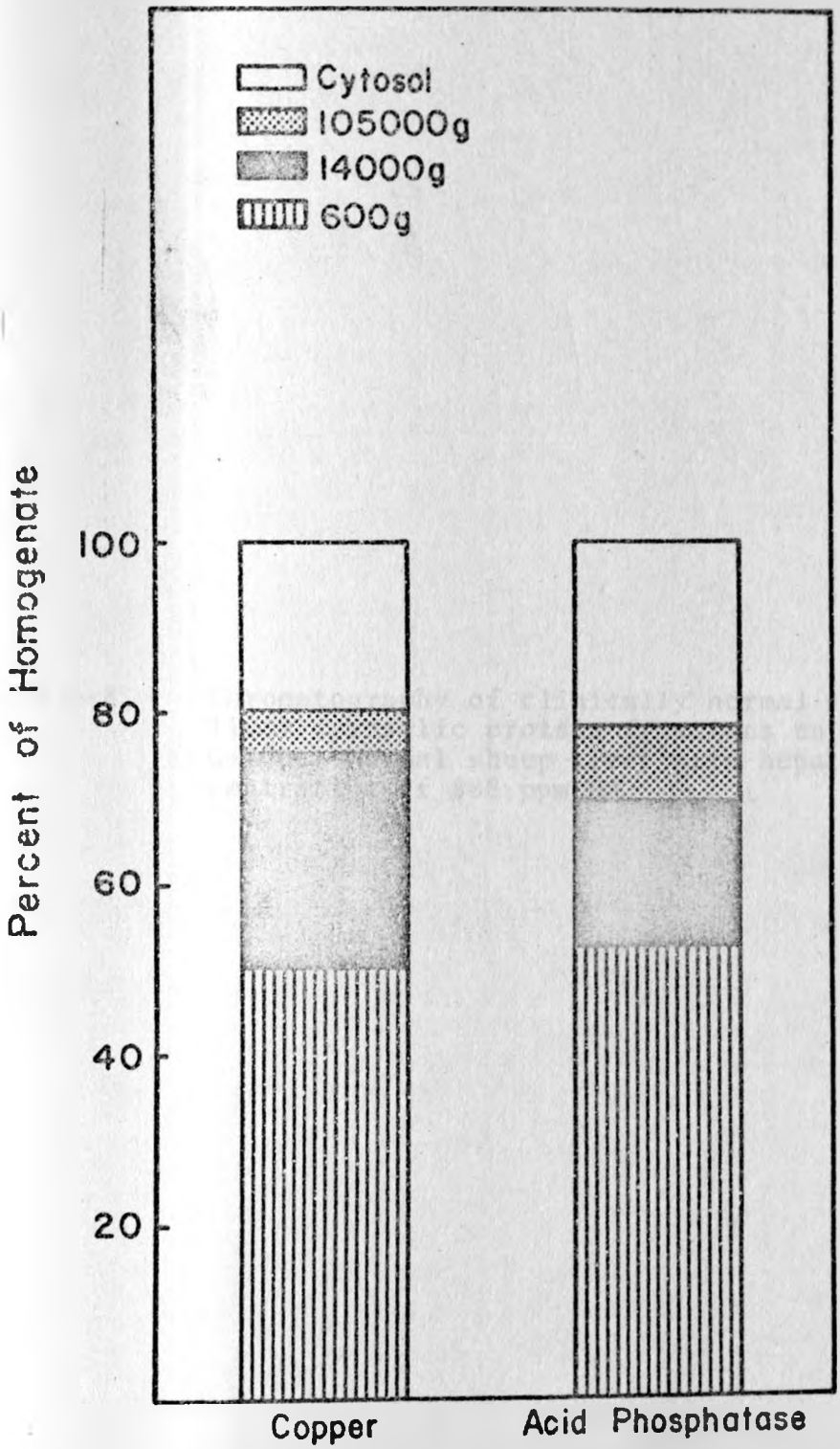

Cytosol
105000g
14000g
600g
Percent of Homogenate
100
80
60
40
20
Copper
Acid Phosphatase

Figure 3-8. Chromatography of clinically normal sheep liver cytosolic protein fractions on Sephadex G-100. Normal sheep liver with hepatic concentration of 888 ppm DM.

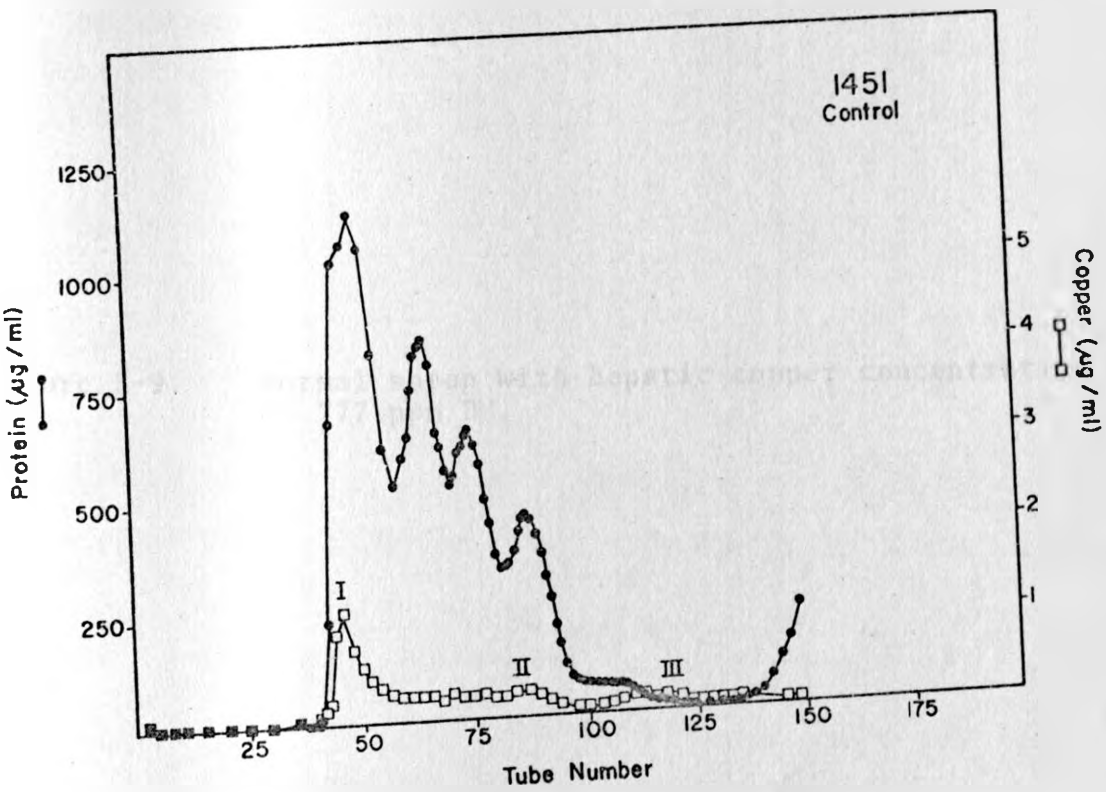

1451
Control
Protein (µg / ml)
Copper (µg / ml)
1250
1000
750
500
250
5
4
3
2
1
I
II
III
25
50
75
100
125
150
175
Tube Number

Figure 3-9. Normal sheep with hepatic copper concentration of 777 ppm DM.

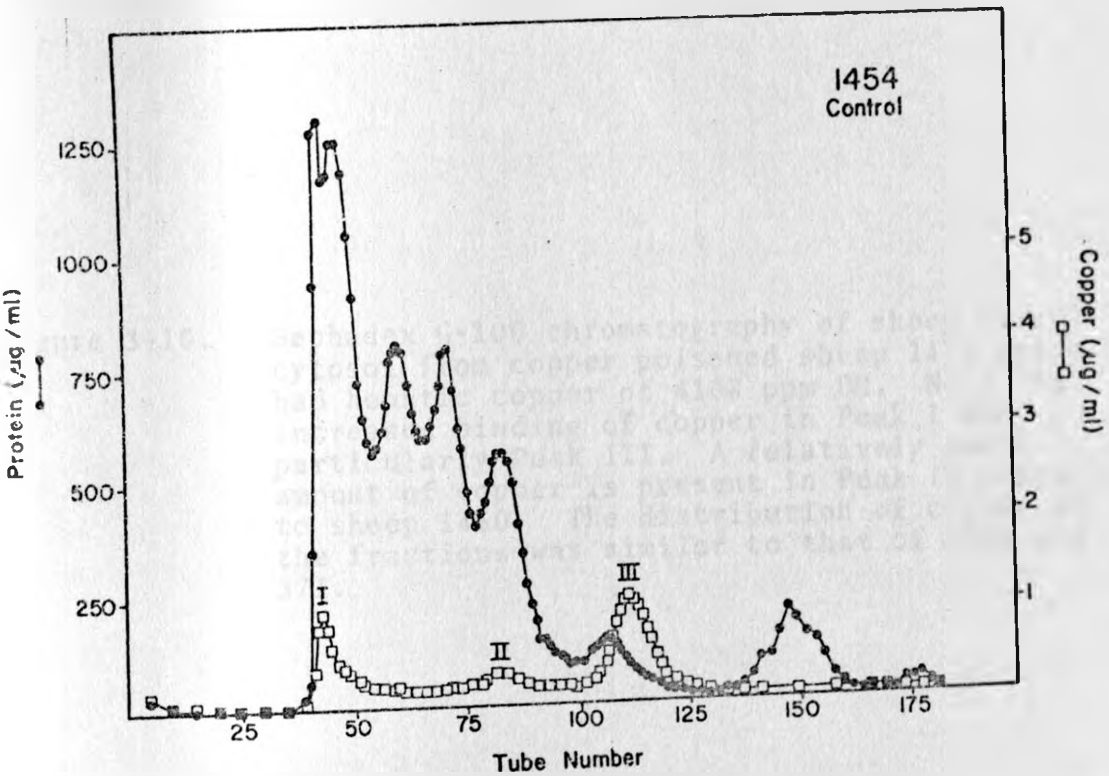

1454
Control
Protein (µg / ml)
Copper (µg / ml)
Tube Number
I
II
III
1250
1000
750
500
250
5
4
3
2
1
25
50
75
100
125
150
175

Figure 3-10. Sephadex G-100 chromatography of sheep liver cytosol from copper poisoned sheep 1473 which had hepatic copper of 4108 ppm DM. Note the increased binding of copper in Peak I and particularly Peak III. A relatively small amount of copper is present in Peak IV compared to sheep 1450. The distribution of copper in the fractions was similar to that of 1456 and 371.

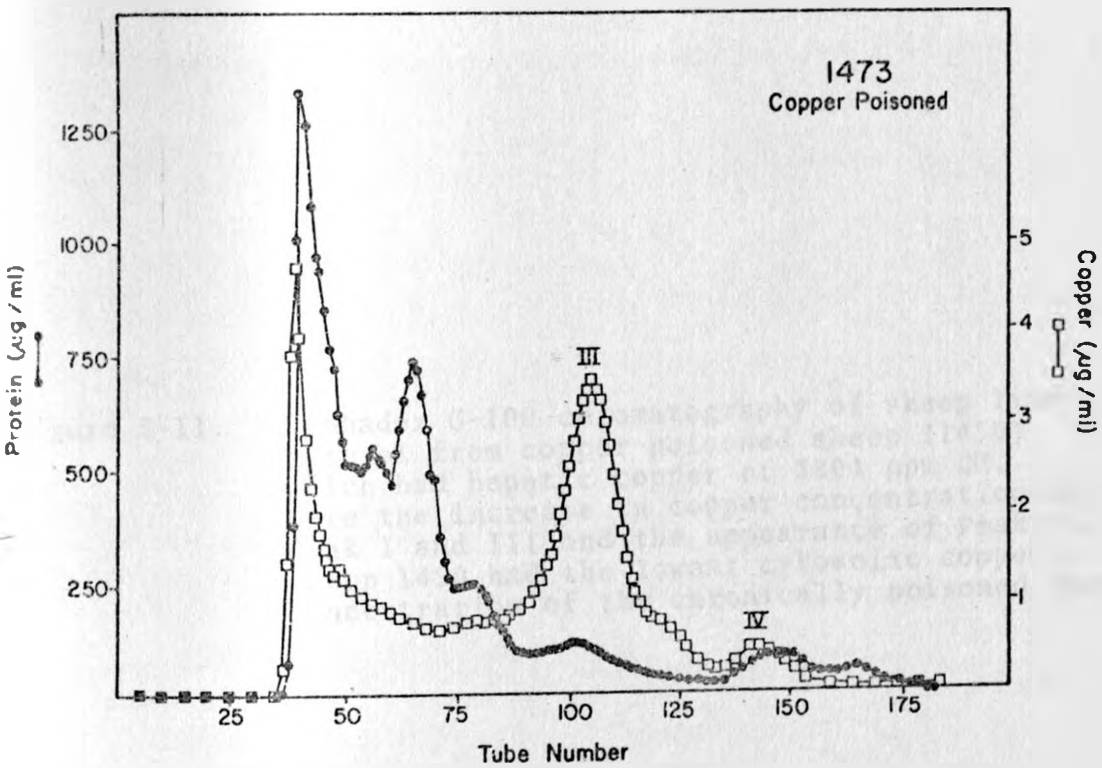

1473
Copper Poisoned
Protein (µg / ml)
Copper (µg / ml)
1250
1000
750
500
250
5
4
3
2
1
III
IV
25
50
75
100
125
150
175
Tube Number

Figure 3-11. Sephadex G-100 chromatography of sheep liver cytosol from copper poisoned sheep (1450) which had hepatic copper of 3804 ppm DM. Note the increase in copper concentration in Peak I and III and the appearance of Peak IV. Sheep 1450 had the lowest cytosolic copper concentration of the chronically poisoned sheep.

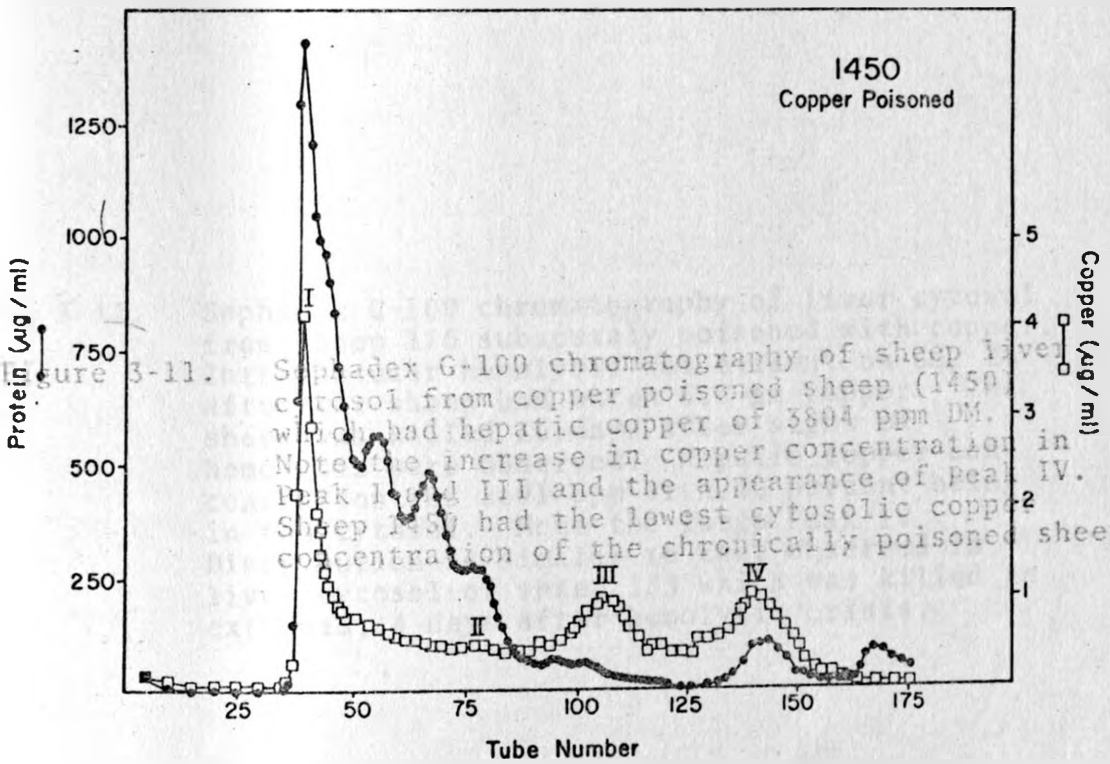
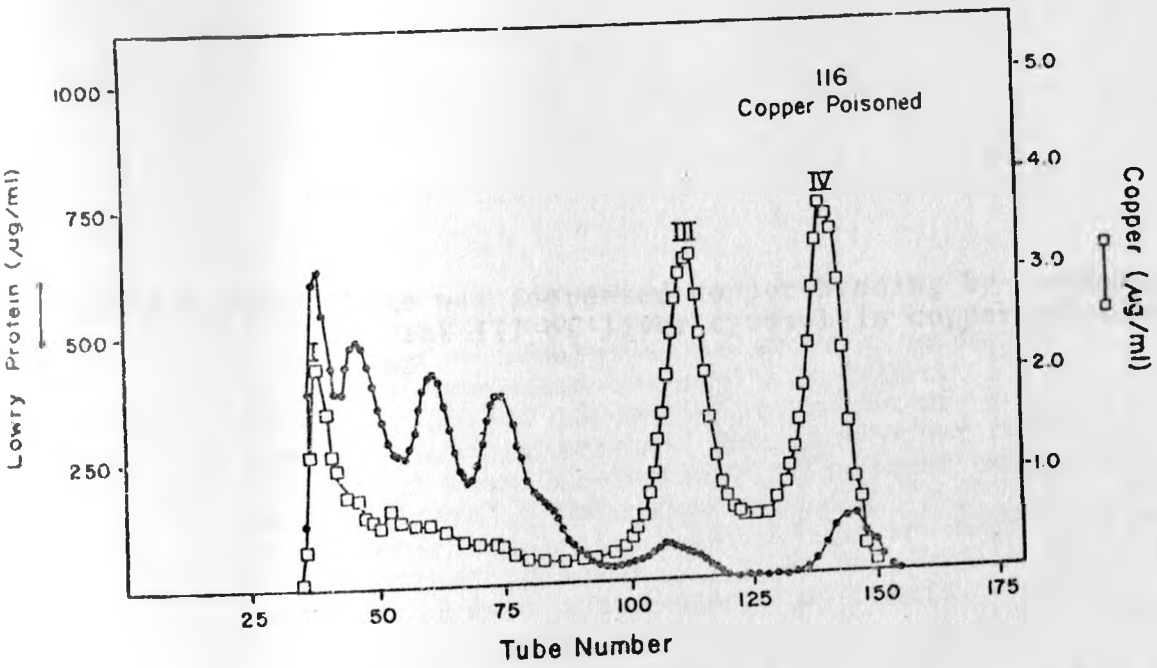


Figure 3-11. Sephadex G-100 chromatography of sheep liver cytosol from copper poisoned sheep (1450) which had hepatic copper of 3804 ppm DM. Note the increase in copper concentration in Peak I and III and the appearance of peak IV. Sheep 1450 had the lowest cytosolic copper concentration of the chronically poisoned sheep.

Figure 3-12. Sephadex G-100 chromatography of liver cytosol from sheep 116 subacutely poisoned with copper. Intravascular hemolysis was evident on day 26 after the sheep had eaten 6.0 gm copper. The sheep was killed 16 days after signs of hemolysis were observed. Hepatic copper concentration was 2601 ppm with 40 percent being in the cytosol. Note the large Peak IV. Distribution is similar to that observed in liver cytosol of sheep 133 which was killed in extremis, 4 days after hemolytic crisis.


116
Copper Poisoned
I
III
IV
1000
750
500
250
5.0
4.0
3.0
2.0
1.0
25
50
75
100
125
150
175
Tube Number
Lowry Protein (µg/ml)
Copper (µg/ml)

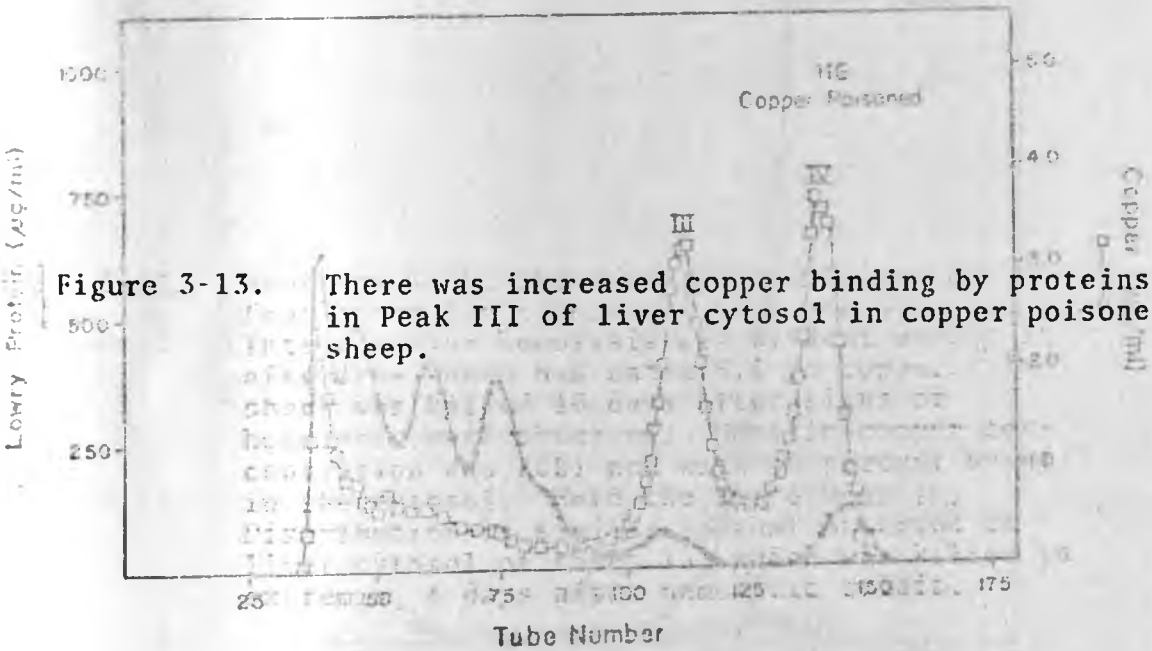


Figure 3-13. There was increased copper binding by proteins in Peak III of liver cytosol in copper poisoned sheep.

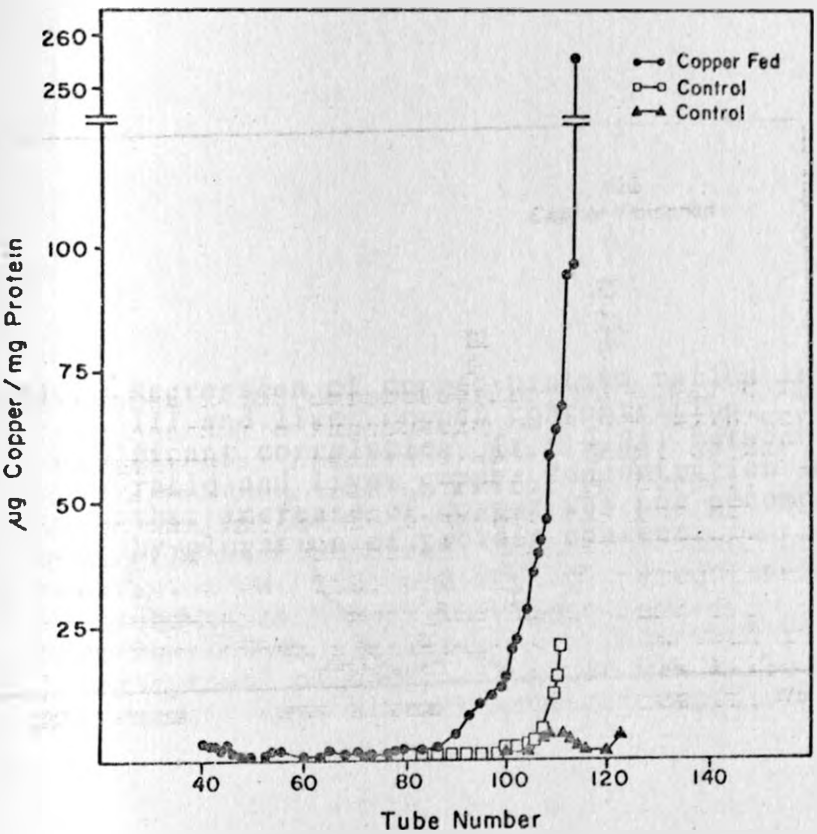

260
250
100
75
50
25
µg Copper / mg Protein
Copper Fed
Control
Control
40
60
80
100
120
140
Tube Number

Figure 3-14. Regression of copper-protein ration in Peak III and liver copper concentration. The significant correlation ($r = 0.84$) between the ratio and liver copper concentration implies that increase of copper was not accompanied by elevation of protein content.

Peak III
Copper: Protein Ratio
(µg Copper / mg Protein)
50
40
30
20
10
1000
2000
3000
4000
Liver Copper (µg/gm DM)

CHAPTER IV

HEMOLYTIC ANEMIA IN EXPERIMENTAL CHRONIC COPPER POISONING OF SHEEP

Introduction

Although in chronic copper poisoning of sheep there is progressive accumulation of copper in the liver, affected sheep remain clinically normal until an acute hemolytic crisis occurs. During the hemolytic crisis, blood copper concentration may increase significantly above normal. There is a rapid decrease in reduced glutathione (GSH) levels which is accompanied by Heinz body formation. A rapid drop in hematocrit occurs within 24 hours. There is methemoglobinemia, hemoglobinemia and hemoglobinuria (Todd, 1969).

The mechanism of the hemolytic anemia in chronic copper poisoning is not clearly understood. The condition has been compared to drug induced hemolytic anemia in human patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Todd, 1969). Both conditions result in a decrease of GSH which is essential for integrity and survival of the erythrocyte (Jaffe, 1970). Though sheep red blood cells have lower activity of G6PD than erythrocytes of normal human beings (Salvidio et al., 1963), they are as resistant to the action of oxidative drugs as human erythrocytes (Smith, 1968). Oxidation of glucose through the pentose phosphate pathway provides NADPH necessary for reducing oxidized glutathione (GSSG) through the pathway shown in Figure 4-1 (Ganther et al., 1976).

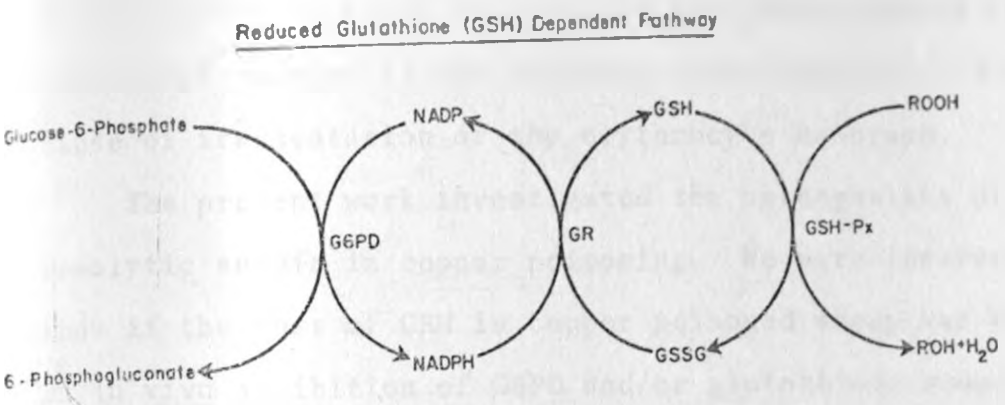


Figure 4-1. Reduced glutathione dependent pathway.

Deficiency and/or inhibition of glutathione peroxidase (GSH-Px) can lead to dangerous accumulations of hydroperoxides with consequent oxidative damage to hemoglobin and red cell membranes leading to hemolysis (Ganther et al., 1976). Thompson and Todd (1970) and Kosower et al. (1969) have observed that GSH oxidation to GSSG does not necessarily result in hemolysis. It is possible, therefore, that GSH loss coupled with inhibition of GSH-Px might lead to dangerous accumulation of peroxides and subsequent hemolysis of erythrocytes.

Another possible explanation for the acute hemolytic anemia of chronic copper poisoning is the accelerated destruction of erythrocytes containing Heinz bodies (Soli and Nafstad, 1976). Intravascular destruction of erythrocytes predominated over their removal by the reticulo-endothelial system (RES). Electron microscopic work revealed interruptions and loss of the laminar structure of erythrocyte plasma membrane where the Heinz bodies were attached (Soli and Nafstad,

1976). This was taken as evidence that Heinz bodies induced structural changes in the membrane which would be a direct cause of fragmentation of the erythrocyte membrane.

The present work investigated the pathogenesis of the hemolytic anemia in copper poisoning. We were interested to know if the loss of GSH in copper poisoned sheep was due to an *in vivo* inhibition of G6PD and/or glutathione reductase (GR) and what role, if any, GSH-Px had to play in increased erythrocyte hemolysis during chronic copper poisoning.

In addition, erythrocyte morphology was examined before and during the hemolytic crisis using the scanning electron microscope to discern structural changes in the erythrocyte membrane.

Materials and Methods

Details on animal feeding and treatment were given in Chapter II of this thesis under the heading "Chronic Copper Feeding Experiment".

Blood Collection

The sheep were bled weekly for erythrocyte enzyme analyses and for reduced glutathione (GSH), hematocrit and hemoglobin determinations. Bleeding was done daily when there was evidence of hemolytic anemia. Blood samples were obtained by jugular vein puncture using heparinized vacuum tubes and disposable needles. Venous blood for total leukocyte and erythrocyte counts and for differential leukocyte counts was

obtained in vacuum tubes containing sodium ethylenediaminetetracetate (EDTA). Blood for scanning electron microscopy was also obtained at weekly intervals using vacuum tubes and heparin as anticoagulant.

Processing of Erythrocytes for Enzyme Analysis

All enzyme assays were performed on blood samples obtained the day of analysis. Heparinized blood was centrifuged[a] at 2000 RPM for 15 minutes at 2C. Plasma was harvested and stored at -20C. The buffy coat was discarded. The packed erythrocytes were washed twice with four volumes of ice cold isotonic saline. The washed red blood cells were stored in test tubes covered with parafilm and placed in an ice bath.

The hemolysate for G6PD analysis was prepared by adding 0.2 ml of packed washed red cells to 6 ml of hemolyzing solution containing 10 uM NADP, 7 mM beta-mercaptoethanol and 2.7 mM EDTA buffered to pH 7.0. The mixture was thoroughly mixed and placed in an ice bath.

The GR hemolysate was prepared by adding 0.2 ml of packed washed red cells to 3.8 ml of distilled water. The mixture was mixed and stored in an ice bath until analyzed.

The GSH-Px hemolysate was prepared by adding 0.2 ml of packed erythrocytes to 3.8 ml of hemolyzing mixture composed of 100 mg of NaCN and 300 mg of $K_2Fe(CN)_6$ per liter of distilled water. The hemolyzing solution was made in advance

[a] Model J21B, Beckman Instruments, Inc., Fullerton, Cal. 92631.

and stored at room temperature.

Analytical Procedures

Hematocrit. Hematocrit was determined in a high speed microhematocrit centrifuge.[a]

Hemoglobin (Hb). Whole blood and hemolysate hemoglobin was determined using a cyanmethemoglobin method.[b] Optical density (OD) measurements were made at 540 nm[c] and hemoglobin concentration (g/dl) was determined from a standard curve.

Reduced Glutathione. GSH was determined utilizing 5, 5' Dithiobis-(-2-nitrobenzoic acid) (DTNB).[d] This substrate reacts with GSH to form a highly colored yellow ion.

$$2GSH + DTNB \text{ (colorless)} \longrightarrow GSSG \times 2TNB^{-} \text{ (yellow)}$$

Heparinized blood, 0.2 ml, was added to 2.3 ml of distilled water to release glutathione by rupture of the erythrocyte membrane. Protein precipitation was accomplished by the addition of 2.5 ml of glutathione reagent consisting of 71 mM sodium tungstate, 84 mM sulfuric acid and 1 mM DTNB. The solution was mixed and centrifuged at 2000 RPM for 15 minutes.[e]

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[a] Model MB, International Equipment Co., Needham Heights, Mass. 02194.

[b] Cyanmethemoglobin method, Hycel, Inc., Houston, Tex. 77000.

[c] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[d] Glutathione "Erythrozyme" Assay, Princeton Biomedix, Inc., Princeton, N.J. 08540.

[e] Model J21B, Beckman Instruments, Inc., Fullerton, Cal. 92631.

OD of 2.5 ml of clear supernatant was measured[a] against water at 412 nm in a cuvette with 1 cm light path (OD_1). Contents of the cuvette were transferred to a tube containing 150 mg of dry phosphate buffer. The solution was thoroughly mixed and the reaction proceeded because the acid supernatant had been neutralized by the buffer (final pH - 7.0). After precisely 5 minutes at room temperature the OD at 412 nm was read (OD_2) against water. Δ OD was obtained by subtracting OD_1 from OD_2. From the hematocrit determined for the blood, GSH concentration was calculated using the formula:

$$\text{mgGSH/100 ml erythrocytes} = \frac{OD}{11000} \times \frac{5}{0.2} \times \frac{100}{PCV} \times 307.3 \times 100$$

where 11,000 = extinction coefficient of DTNB at 412 nm.

5 = final volume of solution (ml).

0.2 = volume of blood originally hemolysed (ml).

100/PCV = factor to convert blood sample volume to red cell volume.

307.3 = molecular weight of GSH.

100 = factor to convert results from milliliter to deciliter.

Glucose-6-Phosphate Dehydrogenase (G6PD). G6PD was assayed by a modified World Health Organization method.[b] The

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[a] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[b] WHO Technical Report Series No. 366, p. 30, 1967.

reagents for this assay were prepared in advance, stored in 5 ml aliquots at -20C and thawed at room temperature immediately prior to use. The buffer solution contained 2.0 mM NADP, 0.1 M magnesium chloride and 1.0 M Tris-HCl adjusted to pH 8.0. The substrate solution contained 6 mM glucose-6-phosphate (G6P). An 0.85 ml volume of the buffer and 0.05 ml of hemolysate were added to a cuvette with 1 cm light path and allowed to equilibrate at 37C for 5 minutes. The reaction was initiated by adding 0.1 ml of the G6P solution to the reaction mixture. The change in OD at 340 nm was recorded against a reagent blank.[a] The ΔOD per minute was calculated and the activity expressed in IU/gm as recommended by Beutler (1971).

Glutathione peroxidase (GSH-Px). GSH-Px was assayed according to the method of Beutler (1971). GSH-Px catalyzes the oxidation of GSH to GSSG by hydrogen peroxide (H_2O_2). The rate of formation of GSSG is measured by means of a GR reaction. Into a cuvette of 1 cm light path was added 0.1 ml of 1 M potassium phosphate buffer pH 7.0; 0.01 ml of 0.1 M GSH; 0.02 ml of 0.2 M EDTA; 0.1 ml of GR (10 U/ml); 0.01ml of 0.4 M sodium azide; 0.1 ml of 2 mM NADPH; 0.02 ml of 1 in 20 ferricyanide-cyanide hemolysate and 0.63 ml distilled water. The mixture was equilibrated at 37C for ten minutes and the reaction started by adding 0.01 ml of 10 mM H_2O_2. The drop in OD

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[a] Model 25, Beckman Instruments, Inc.; Fullerton, Cal. 92631.

[b] WHO Technical Report Series No. 366, p. 30, 1967.

was recorded at 340 nm against a reagent blank.[a] The reaction without hemolysate, using the hemolyzing solution alone was run to correct for the non-enzymatic oxidation of GSH. Δ OD per minute of non-enzymatic reaction was subtracted and activity expressed in IU/g Hb.

The concentration of H_2O_2 was adjusted monthly, using the molar extinction coefficient. The OD of 0.9 ml of 0.1 M phosphate buffer pH 7.0 was measured at 230 nm (OD_1). To this buffer was then added 0.1 ml of a 1 in 10 dilution of 3 percent H_2O_2 and OD measured again (OD_2). The H_2O_2 concentration (C) of the 0.3 percent H_2O_2 solution was 141 (OD_2 - OD_1) mM. For the assay 1 ml of 0.3% H_2O_2 was diluted to C/10 ml with distilled water.

H_2O_2, NADPH, GSH and GR (10 U/ml) were prepared fresh daily. All reagents were stored on ice before the analysis was done.

Hematology. Total erythrocyte and leukocyte counts were made automatically on a Coulter Counter.[b] Blood smears made from EDTA blood were stained with Wright's stain or new methylene blue stain for differential counting.

Erythrocyte scanning. Blood for scanning was obtained in heparinized vacuum tubes and fixed within ten minutes according to the method of Bessis (1973). Animals were bled weekly during the copper feeding period and daily when signs

[a] Model 25, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[b] Coulter Counter Electronics, Hialeah, Fla. 33000.

of hemolysis were noted. Blood was diluted 1 in 20 in freshly made 1 per cent glutaraldehyde solution in Eagle's modified medium pH 7.4. Agitation of the tube as blood was added insured rapid mixing to avoid clumping of erythrocytes. The erythrocytes were allowed to fix for 30 minutes at room temperature during continuous agitation in a water bath shaker.[a]

After fixation, cells were washed two times with Eagle's solution and centrifuged at 600 RPM x 5 minutes each time.[b]

The erythrocytes were post-fixed in freshly made 0.5 percent osmium tetroxide solution in Eagle's medium pH 7.4, for 60 minutes. They were washed three times with distilled water to remove the osmium salt crystals and dehydrated in 50, 70, 80, 95 and 100 percent alcohol, for five minutes in each alcohol in that order. Dehydrated cells were suspended in 2 ml propylene oxide. A drop of this suspension placed on a coverslip dried immediately. The coverslip was fixed to a stub and sample coated by evaporating a palladium-gold wire while the sample was rapidly rotated.[c] The coated cells were scanned and photographed under a scanning electron microscope.[d]

Results

Hematology

Erythrocyte parameters. There was a slight decrease in

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[a] Model 2156, American Optical Corp., Buffalo, N.Y. 14215.

[b] Model GLC-2, Ivan Sorvall, Inc., Norwalk, Conn. 06850.

[c] Model 306, Edwards High Vacuum Coater, Sussex, England.

[d] Model HHS-2R, Hitachi, Ltd., Tokyo, Japan.

the hematocrit in all the copper supplemented sheep throughout the experimental period followed by a precipitous decrease during the hemolytic crisis (Fig. 4-2 and 4-3). The mean value of copper poisoned sheep 4 to 10 days before the crisis was 30.4 ± 1.7% (± SEM). The PCV values for the control sheep varied between 26.5 and 37.5% (32.5 ± 0.36) over the experimental period (Table 4-1). The mean PCV of the copper poisoned sheep at the onset of the hemolytic crisis was 21.3 ± 2.6 per cent and was 14.5 ± 1.8, 4 to 10 days following the crisis. Both values were significantly below those of control sheep ($p < 0.05$). Total hemoglobin concentrations followed the same pattern as the PCV (Fig. 4-2). Recovery in sheep 1473 and 1450 from the first hemolytic crisis was followed by a gradual increase of hematocrit and hemoglobin concentration before the second crisis occurred (Fig. 4-2).

Heinz bodies were detectable on the day before the crisis but were found in large numbers during the hemolytic episode (Fig. 4-4).

During and immediately after hemolytic crisis, blood films demonstrated anisocytosis, poikilocytosis and polychromasia (Fig. 4-5).

Recovery from a hemolytic episode was accompanied by reticulocytosis as shown in Table 4-2. The mean reticulocyte count on the day of hemolytic crisis was 2.0 percent which increased to 8.4 percent on the second day and 12.4 percent on the fifth day of the crisis. Reticulocytosis was accompanied by an increase in mean corpuscular volume (MCV) as is

evident from Table 4-2.

Leukocyte parameters. Hemolytic crisis was accompanied by leukocytosis which was mainly due to an increase in neutrophils (Table 4-3).

Reduced Glutathione (GSH)

GSH levels showed a progressive decrease in the copper fed sheep during copper accumulation (Fig. 4-1). Mean for the control sheep during this period was 64.0 ± 2.13 mg/100 ml red blood cells (RBC) (Range 43.3 to 103.9) (Table 4-4). GSH in the copper poisoned sheep was 53.4 ± 4.4 mg/100 ml RBC before the crisis and this dropped to 30 ± 4.8 mg/100 ml RBC during hemolytic crisis. This represented nearly a 47 percent decrease from the pre-hemolytic concentration ($p < 0.05$). Post-hemolytic samples had mean GSH concentrations of 73.5 ± 10.9 (48.9 to 112.8) which was within the normal range (Fig. 4-3).

Glucose-6-phosphate Dehydrogenase (G6PD)

The G6PD activity in the three control sheep ranged from 0.56 to 2.06 IU/g Hb with a mean of 0.95 ± 0.038 (±SEM). The activity of this enzyme fluctuated during the copper feeding period but remained within the normal range (Fig. 4-2). Figure 4-3 and Table 4-4 compare the levels in the periods preceding, during and following the hemolytic crisis. The pre-hemolytic levels (1.41 ± 0.07) were not statistically different from the hemolytic period levels of 1.36 ± 0.14 IU/g Hb. However, the activity of G6PD increased in the period following hemolytic crisis (3.86 ± 0.57 IU/g Hb) (Fig. 4-3) and was statistically

higher than pre-hemolytic values and values of control sheep ($p < 0.01$).

Glutathione Reductase (GR)

The activities of GR in individual copper poisoned sheep are shown in Figure 4-2. There was a slight increase in GR activity in sheep 1473 and 371 in the first week after the start of copper feeding. Otherwise levels remained within normal limits. Activity in the control sheep over the entire experimental period varied from 0.77 to 3.94 IU/g Hb (2.13 ± 0.088). Pre-hemolytic values in copper poisoned sheep were 2.73 ± 0.29 IU/g Hb (1.53 to 3.44), not significantly different from those of the control sheep. Neither were the hemolytic crisis values of 3.12 ± 0.29 IU/g Hb (1.97 to 3.93) different from the control activity. However, the GR activity during the post-hemolytic phase was significantly higher ($p < 0.05$) than both that of control sheep and pre-hemolytic samples. The recorded mean was 6.73 ± 0.68 IU/g Hb (5.4 to 9.28) (Fig. 4-3).

Glutathione Peroxidase (GSH-Px)

As shown in Figure 4-2, the activity of GSH-Px fluctuated during the copper feeding period. The control values ranged from 9.3 to 47.1 IU/g Hb (mean 20 ± 0.89). In the period preceeding the hemolytic crises, GSH-Px activity in copper poisoned sheep was 15.8 ± 1.32 IU/g Hb (range 12.5 to 19.9). This level did not differ from that of the control sheep. Levels recorded during and after hemolytic crises were 22 ±

1.74 (17.5 to 28) and 10.9 ± 3.5 (3.09 to 22.1) IU/g Hb, respectively. Though the mean GSH-Px post-hemolytic activity was significantly lower than that of the controls ($p < 0.05$), Figure 4-3 shows that it was within the normal range.

Erythrocyte Morphology

The morphology of red blood cells was not remarkably changed up to three days before the hemolytic crisis as shown in Figures 4-6 and 4-7. Blood samples scanned on the day before hemolytic crisis revealed a marked alteration in erythrocyte morphology (Fig. 4-8). There was poikilocytosis and many cells developed spicules. An occasional cell had rounded shape (spherocytes). The majority of cells contained lumps pushing on the membrane surface. The protrusions were irregularly distributed over the cell surface. Some red blood cells showed evidence of fragmentation by raised membrane edges which had rough outlines and also by small membrane pieces which appeared to be breaking off.

Figures 4-9 and 4-10 show the morphology of red blood cells on the day of hemolytic crisis. Particularly notable is the marked rounding of the cells (almost 100 percent in the smears taken). The marked spherocytosis tends to mask the lumps observed on the previous day although they can still be seen on some cells. Also very noticeable in these smears is the presence of many erythrocytes with one end rounded and the other end flattened with corrugated membrane edges. Presumably, the latter represents membranes which

have collapsed after loss of stromal contents. The overall picture is that of erythrocyte fragmentation. This fragmentation occurred over the entire surface of the membrane and was not restricted to lumps representing the Heinz bodies pushing on the membrane from inside. Figure 4-10 shows cells at various stages of losing their contents. The final stage is the ghost cell.

Discussion

The onset of a hemolytic crisis was associated with a nearly 50 percent reduction in GSH concentration and appearance of Heinz bodies. This magnitude of GSH loss is common in chronic copper poisoning of sheep (Todd and Thompson, 1963; Thompson and Todd, 1976), in calves (Todd and Thompson, 1964) and in human patients with drug induced hemolytic anemia (Beutler, 1969).

The mechanism of GSH loss in chronic copper poisoning is not clearly understood. The GSH loss in drug sensitive individuals is attributed to a hereditary G6PD deficiency which lowers the capacity of the GSH regenerating pathway. Administration of oxidant drugs such as primaquine result in the observed GSH lowering with associated conditions (Beutler, 1969). Although sheep red blood cells have much lower activity of G6PD and other glutathione dependent pathway enzymes, they have been shown to be as resistant to oxidant stress as human erythrocytes which have much higher quantities of these enzymes (Smith, 1968). There was no recorded inhibition of either G6PD or GR during the hemolytic crisis in this work.

Similar observations have been made by Thompson and Todd (1976) in sheep poisoned with copper. Metz and Sagone (1972) could not demonstrate *in vitro* inhibition of the enzymes, but there are reports which contradict the above findings. Passwell et al., (1970) observed transient inhibition of G6PD during hemolytic episodes in a patient with Wilson's disease. *In vitro* inhibition of G6PD by copper is well documented (Passwell et al., 1970; Fairbanks, 1967). Deiss et al. (1970) also observed *in vitro* inhibition of GR. Despite these *in vitro* observations, Metz and Sagone (1972) recorded a stimulation of the hexose-monophosphate pathway in intact erythrocytes by copper. They warned about the dangers of extrapolating *in vitro* observations to *in vivo* conditions.

The GSH loss can be explained by direct catalytic non-enzymatic oxidation of GSH by copper as reported by Fairbanks (1967) and Metz and Sagone (1972). In addition, copper generates hydrogen peroxide by its reaction with oxy-hemoglobin (Metz and Sagone, 1972). Copper, by its reaction with oxygen, can cause peroxidation of membrane lipids resulting in production of lipid peroxides (Lindquist, 1968). Excess of hydrogen peroxide and lipid peroxides would lead to increased GSH loss through GSH-Px catalyzed oxidation. GSH-Px activity was not altered during hemolytic crisis and it is likely that this enzyme affects GSH levels only through increased catalytic oxidation due to increased oxidative challenge. We conclude that GSH loss in copper poisoning is due to direct oxidation by copper and increased oxidative stress and not due to

inhibition of glutathione dependent pathway enzymes.

The presence of Heinz bodies and hemolysis of erythrocytes is the result of what Kosower et al., (1969) termed "GSH loss catastrophe." The mechanism of destruction of erythrocytes by hemolysis resulting from GSH loss is not well understood. Thompson and Todd (1970) observed that incubation of intact erythrocytes with a copper-containing fraction from the liver of copper poisoned sheep caused GSH oxidation and methemoglobin formation but did not lyse the red cells. Incubation of erythrocytes with lipid extract from the same livers caused lysis. This fraction had high thiobarbituric acid activity which indicated presence of lipid peroxides. Lindquist (1968) also reported that copper increased lipid peroxidation of membranes. Copper has also been observed to increase hydrogen peroxide production (Metz and Sagone, 1972) like other oxidant compounds do (Kosower et al., 1969; Cohen and Hochstein, 1964; Beutler, 1969). H_2O_2 and lipid peroxides, in the absence of the protection afforded by GSH, will lead to destruction of membranes by starting chain reactions of the radicals in the lipid membrane (Kosower et al., 1969; Beutler, 1969).

Cells containing Heinz bodies are also destroyed at an accelerated rate (Jandl et al., 1960, Soli and Nafstad, 1976). The presence of Heinz bodies decreased cell deformability and increased sequestration by the spleen (Rifkind and Danon, 1965). Heinz bodies attached to erythrocyte membranes may induce structural weakness (Soli and Nafstad, 1976) as well

as cause changes in chemical composition of the membrane. The latter may be the cause of intravascular hemolysis observed in copper poisoning (Lubin and Desforges, 1972; Soli and Nafstad, 1976). Soli and Nafstad (1976) observed membrane interruptions in erythrocytes containing Heinz bodies in electron microscopic sections. This evidence was used to support the observed intravascular hemolysis.

The present scanning electron work demonstrated that formation of Heinz bodies immediately before hemolysis was accompanied by marked distortion of erythrocyte membranes with occasional spiculated erythrocytes. Several erythrocytes had membrane interruptions which presented as elevated patches on the surface of erythrocyte membranes. Some cells were rounded which can be taken as evidence of fragmentation (Bessis et al., 1973). Hemolytic crisis was accompanied by increased spherocytosis. Membrane fragmentation was more marked with membrane breaks spread over the entire surface of the erythrocyte.

On the basis of the results from scanning electron microscopy, it would be difficult to speculate on the actual cause of membrane interruptions observed. It is conceivable that membrane injury by peroxides and Heinz body attachment could cause these interruptions which in turn lead to the observed intravascular hemolysis. Non-specific attachment of proteins to erythrocyte membranes caused by copper ions could result in a Coombs positive reaction as observed by Jandl and Simmons (1957), but this would only result in increased sequestration of erythrocytes in the RES.

Summary

The present results show that GSH loss observed during the hemolytic crisis of chronic copper poisoning cannot be attributed to *in vivo* inhibition of G6PD, GR or GSH-Px.

Hemolytic crisis was accompanied by fragmentation of erythrocyte membranes as observed in scanning electron microscopic studies. These membrane breaks and the presence of Heinz bodies caused marked distortion of erythrocytes and were probably a direct cause of observed intravascular hemolysis in chronic copper poisoning.

Table 4-1. Erythrocyte Enzymes, Total Plasma Protein, Hemoglobin and Hematocrit of the Control Sheep in the Chronic Copper Experiment.

	No. of Analyses	Mean ± SEM	Range
GSH Px[a] (IU/g Hb)	59	20 ± 0.89	9.3 - 47.1
G6PD[b] (IU/g Hb)	62	0.95 ± 0.038	0.56 - 2.06
GR[c] (IU/g Hb)	62	2.13 ± 0.088	0.77 - 3.94
GSH[d] (mg/100 ml RBC)	59	64.0 ± 2.13	43.3 - 103.9
Hematocrit (%)	71	32.5 ± 0.36	26.5 - 37.5
Hemoglobin (g/dl)	71	10.7 ± 0.19	7.5 - 12.8
Total protein (g/dl)	69	6.1 ± 0.064	5.0 - 7.3

[a] GSH-Px - Glutathione peroxidase.

[b] G6PD - Glucose-6-phosphate dehydrogenase.

[c] GR - Glutathione reductase.

[d] GSH - Reduced glutathione.

Table 4-2. Mean Reticulocyte Counts (%) and Mean MCV in Blood of Sheep Chronically Poisoned with Copper.

	Control	Days After Hemolytic Crisis 0	2	5
Reticulocyte Count (%)	0	2.0(4)[a]	8.4(3)	12.88(1)
MCV (u^3)	33(4)	35.8(3)	43.3(3)	-

[a] Number of sheep tested.

Table 4-3. Leukocyte Changes in Sheep Undergoing Hemolytic Crisis of Chronic Copper Poisoning.

	Control	Days Before or After Hemolytic Crisis		
		-2	0	2
WBC x 10^3/ul	7.0(4)[a]	7.4(1)	19.6(4)	9.5(3)
Segmented Neutrophils x 10^3/ul	2.6(4)	5.0(1)	8.8(4)	3.8(3)
Lymphocytes x 10^3/ul	5.6(5)	2.5(1)	11.6(4)	6.5(3)

[a] Number of sheep tested. Day 0 represents start of hemolytic crisis.

Table 4-4. Activities of Erythrocyte Enzymes, Values of Reduced Glutathione (GSH) and Hematocrit Before and After Hemolytic Crisis and in 3 Control Sheep (Mean ± SEM).

	GSH-Px IU/gm Hb	G6PD IU/gm Hb	GR IU/gm Hb	GSH mg/100 ml RBC	Hematocrit %
Normal Controls	20.0±0.89 (9.3-36.1)	0.95±.038 (.56-2.06)	2.13±.088 (0.77-3.94)	64.0±2.13 (43.3-103.9)	32.5±0.36 (26.5-37.5)
Pre-hemolytic Crisis	15.8±1.32 (12.5-19.9)	1.41±0.07 (1.17-1.57)	2.73±0.29 (1.53-3.45)	53.4±5.4 (27.8-67.5)	30.4±1.7 (24.0-36.0)
Hemolytic Crisis	22.0±74 (17.5-28.0)	1.36±0.14 (0.80-1.72)	3.12±0.29 (1.97-3.93)	30.0±4.8 (11.5-38.0)	21.3±2.6 (9.0-28.0)
Post-hemolytic Crisis	10.9±4.5 (3.09-22.1)	3.86±0.57 (2.14-5.36)	6.73±0.68 (5.4-9.28)	73.5±10.9 (48.9-112.8)	14.5±1.8 (11.5-21.0)

Figure 4-2. Details of changes in activities of pentose phosphate shunt enzymes, PCV and hemoglobin in chronically poisoned sheep.

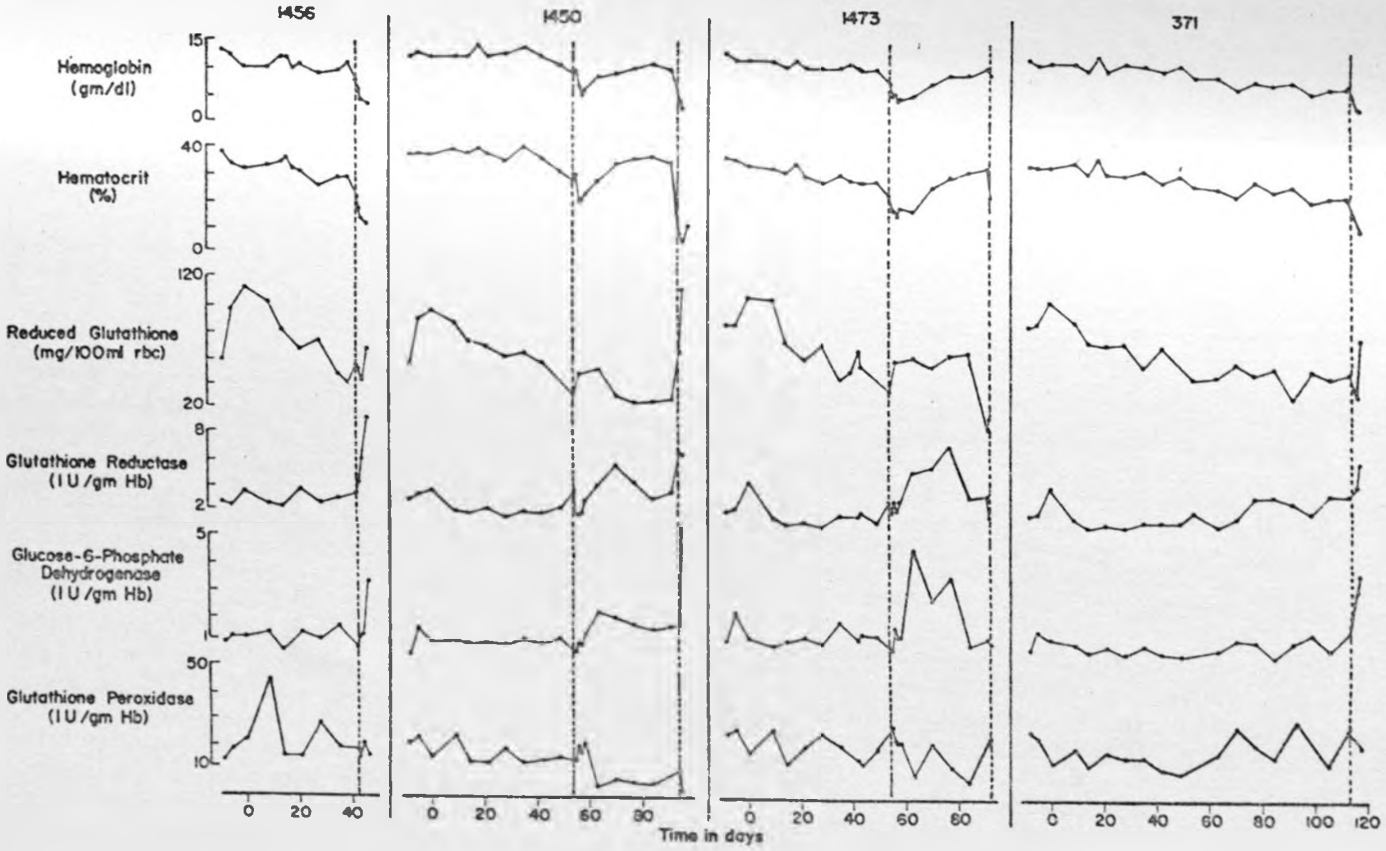

1456
1450
1473
371
Hemoglobin (gm/dl)
15
0
Hematocrit (%)
40
0
Reduced Glutathione (mg/100ml rbc)
120
20
Glutathione Reductase (I U/gm Hb)
8
2
Glucose-6-Phosphate Dehydrogenase (I U/gm Hb)
5
1
Glutathione Peroxidase (I U/gm Hb)
50
10
0 20 40
0 20 40 60 80
0 20 40 60 80
0 20 40 60 80 100 120
Time in days

Figure 4-3. Summary of the changes of the activities of GSH-Px, G6PD, GR and changes in GSH and hematocrit in the period around the hemolytic crisis.

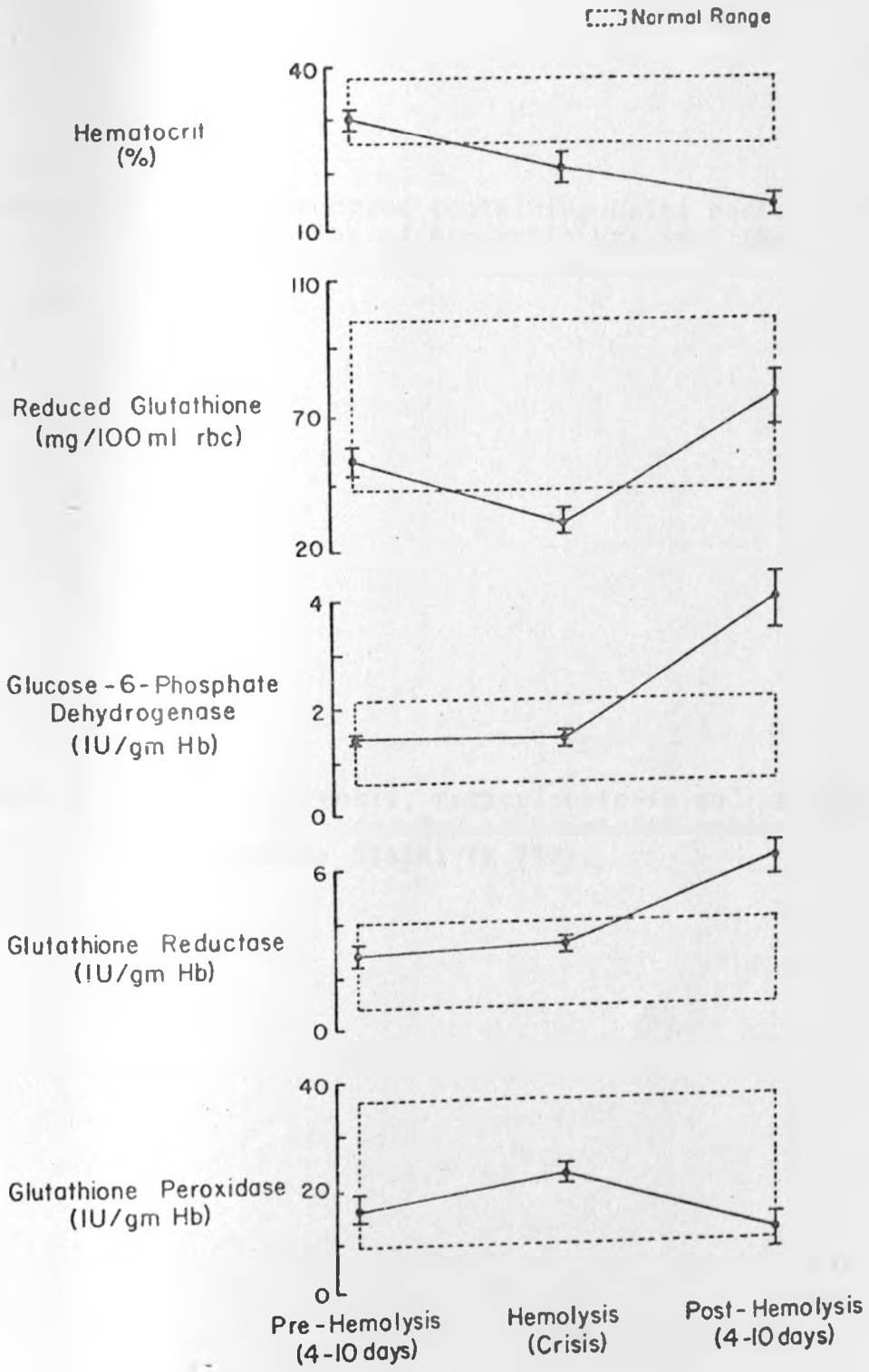

Normal Range
Hematocrit (%)
40
10
Reduced Glutathione (mg/100 ml rbc)
110
70
20
Glucose-6-Phosphate Dehydrogenase (IU/gm Hb)
4
2
0
Glutathione Reductase (IU/gm Hb)
6
0
Glutathione Peroxidase (IU/gm Hb)
40
20
0
Pre-Hemolysis (4-10 days)
Hemolysis (Crisis)
Post-Hemolysis (4-10 days)

Figure 4-4. Erythrocytes containing Heinz bodies at the beginning of hemolytic crisis. (New Methylene Blue) (x 520).

Figure 4-5. Anisocytosis, reticulocytosis and polychromasia on the third day of hemolytic crisis. (Wrights Stain) (x 700).

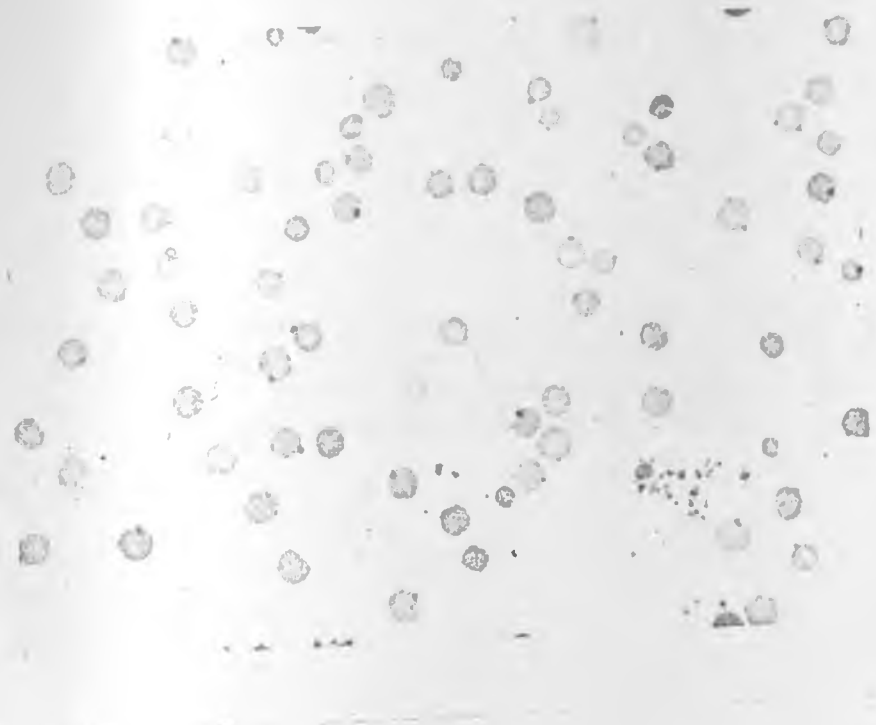


Figure 4-4

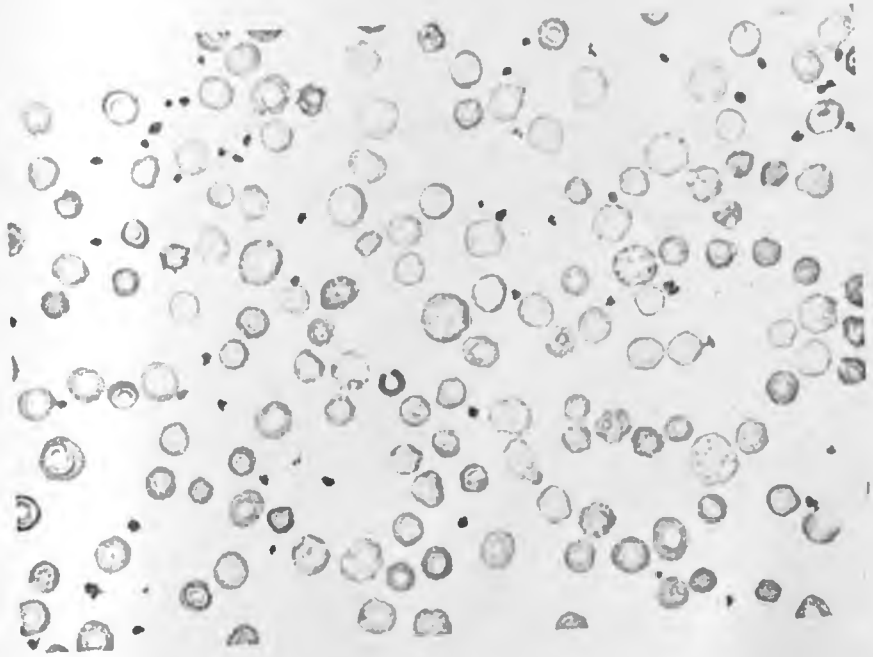


Figure 4-5

Figure 4-6. Scanning electron micrograph (SEM) of erythrocytes in copper poisoned sheep (371) three days before the hemolytic crisis. Note the normal biconcave shape (x 7000).

Figure 4-7. SEM of erythrocytes from a normal sheep. The shape and size of the erythrocytes are the same as observed in sheep 371, three days before hemolytic crisis (x 7000).

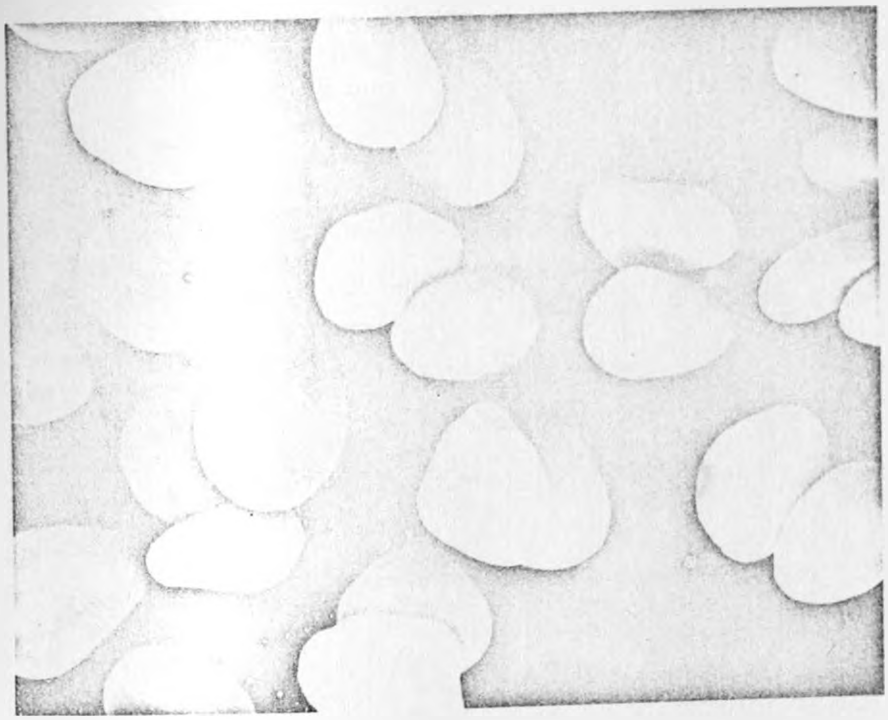


Figure 4-6

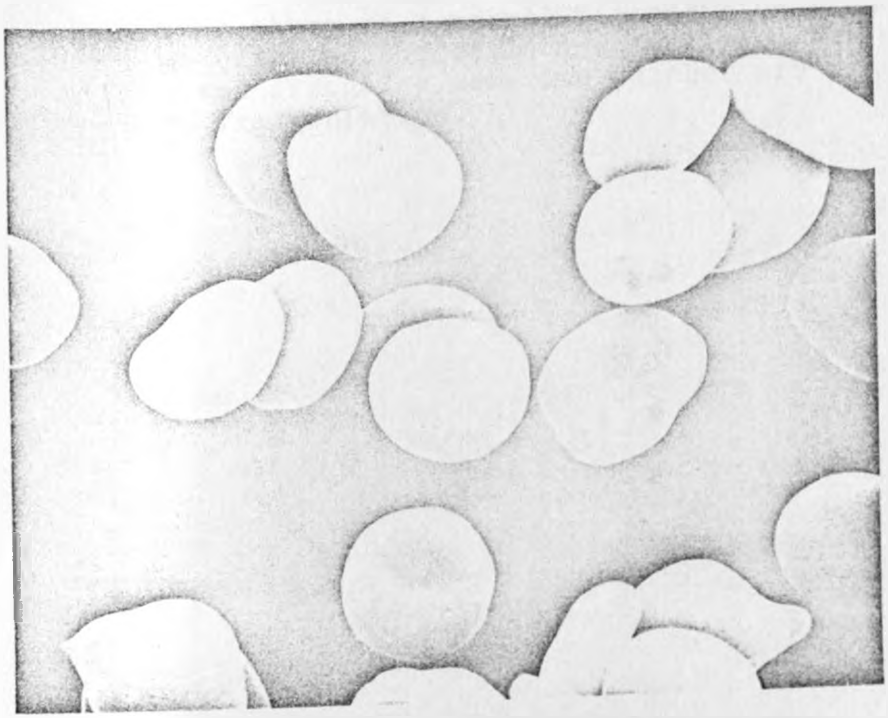


Figure 4-7

Figure 4-8. SEM of the erythrocytes on the day before hemolytic crisis. There is marked distortion with swellings and spicules. Protruding pieces of membrane are the result of fragmentation. (a) x 3000, (b) x 7000 and (c) x 10,000. In 4-8c there is a normal biconcave cell but the majority of the cells are swollen. The elevated irregular membrane patches are evidence of fragmentation.

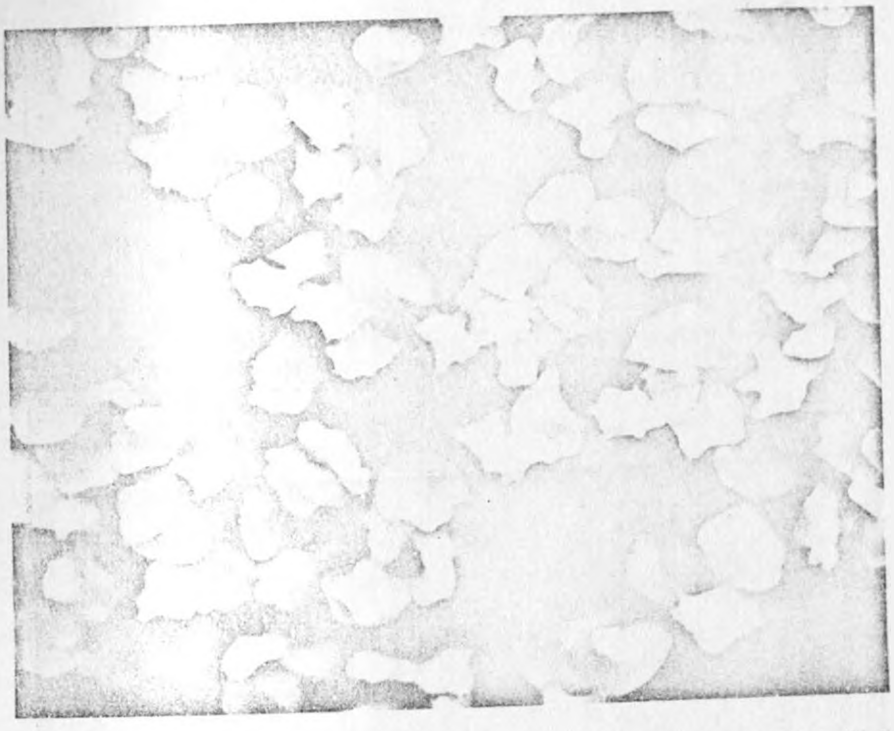


Figure 4-8a

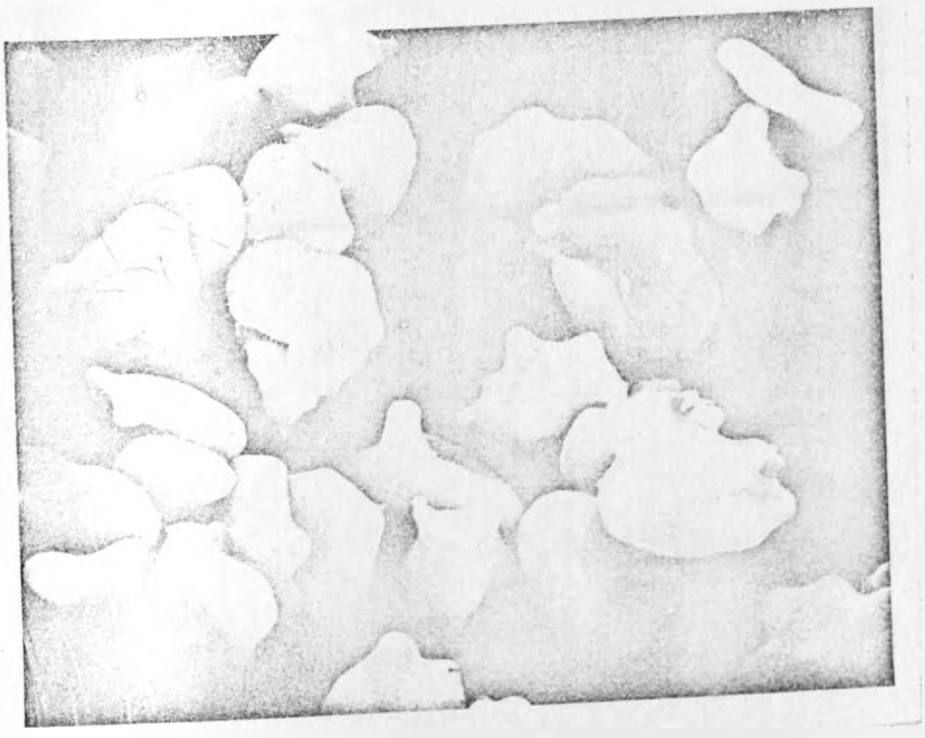


Figure 4-8b

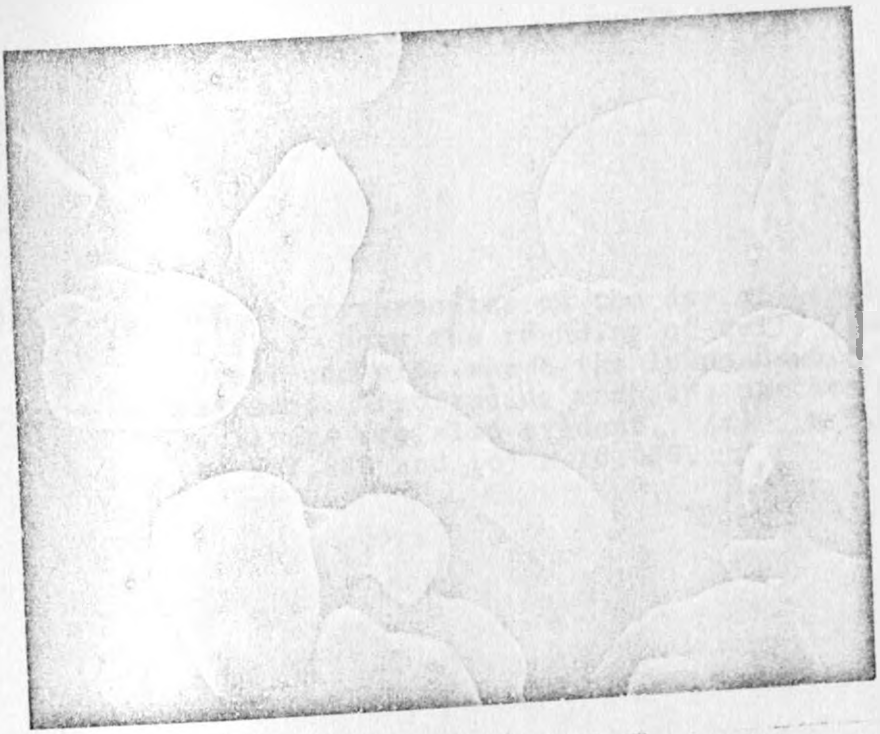


Figure 4-8c

Figure 4-9. SEM of erythrocytes on the day of hemolytic crisis. Note the rounding of cells (spherocytes) and pits where the lumps have been extruded. Protruding membrane patches and swellings are also evident. (a) x 3,000, (b) x 7,000 and (c) x 10,000.

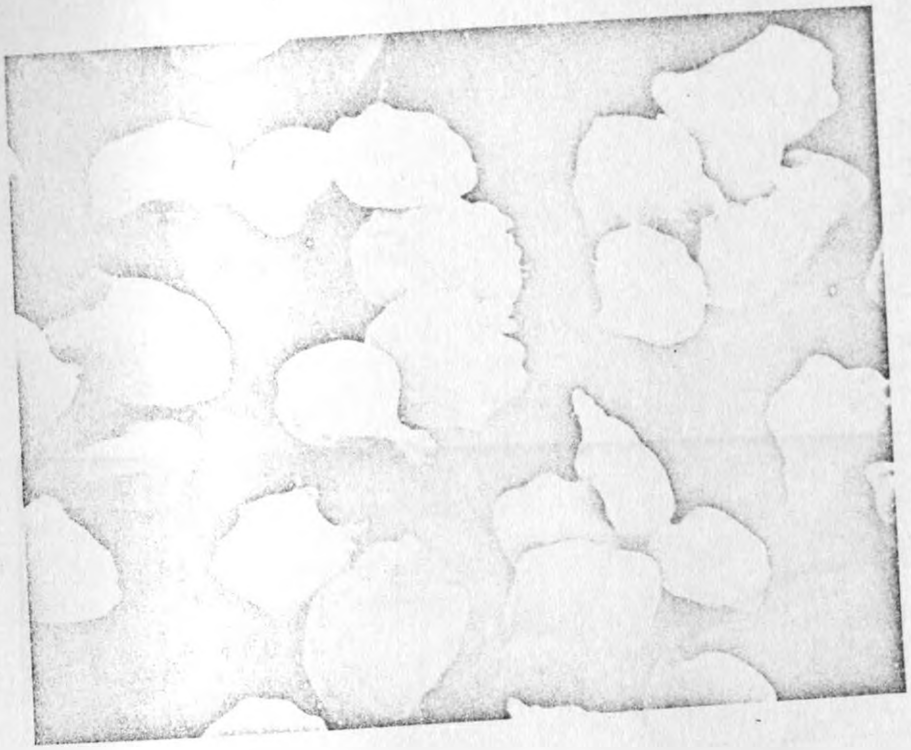


Figure 4-9a

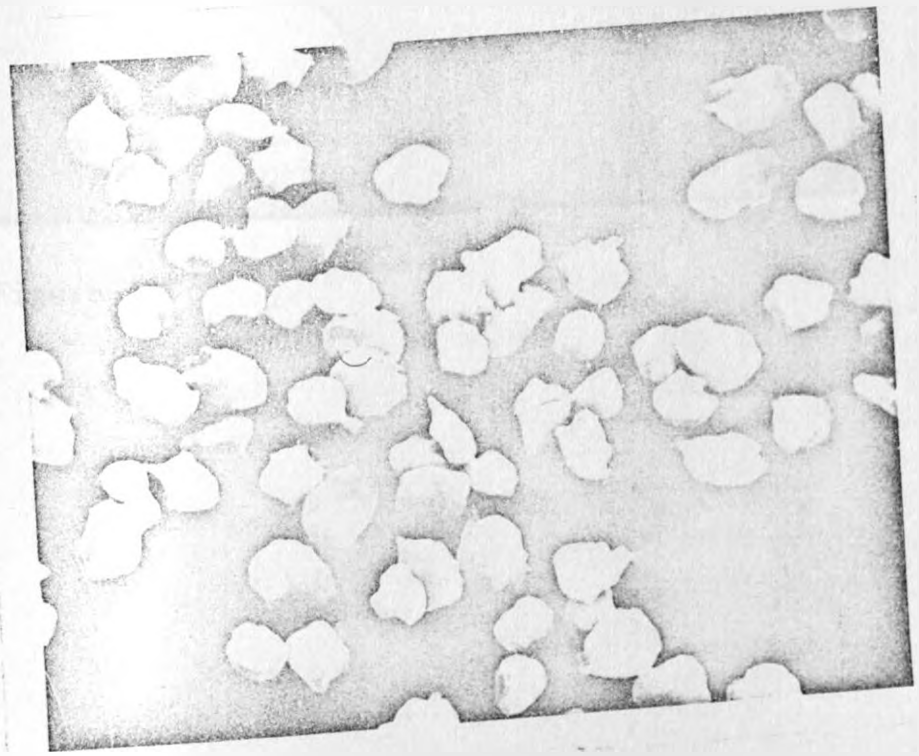


Figure 4-9b

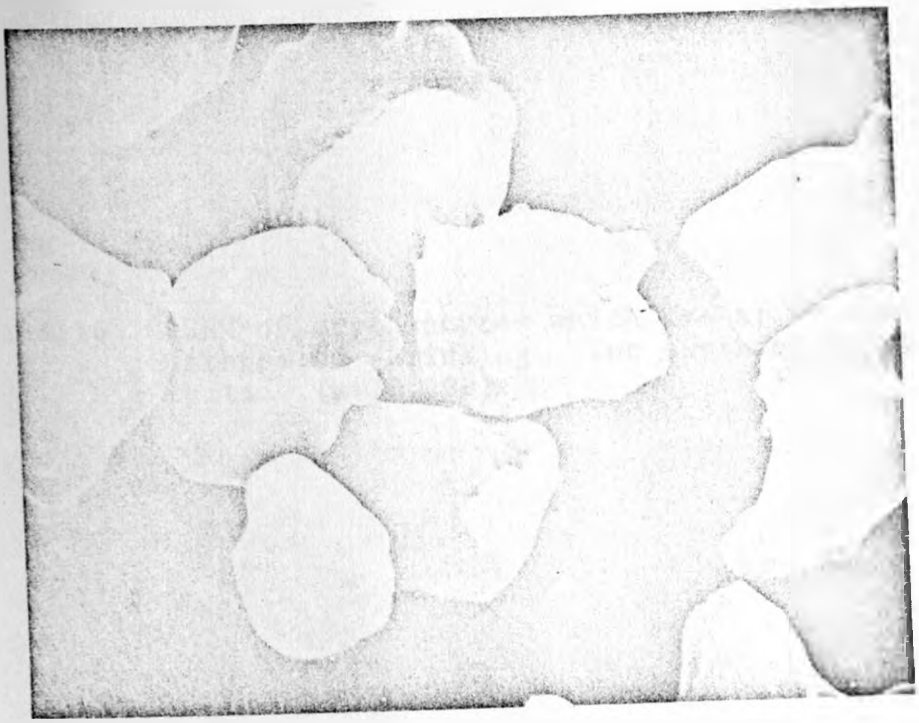


Figure 4-9c

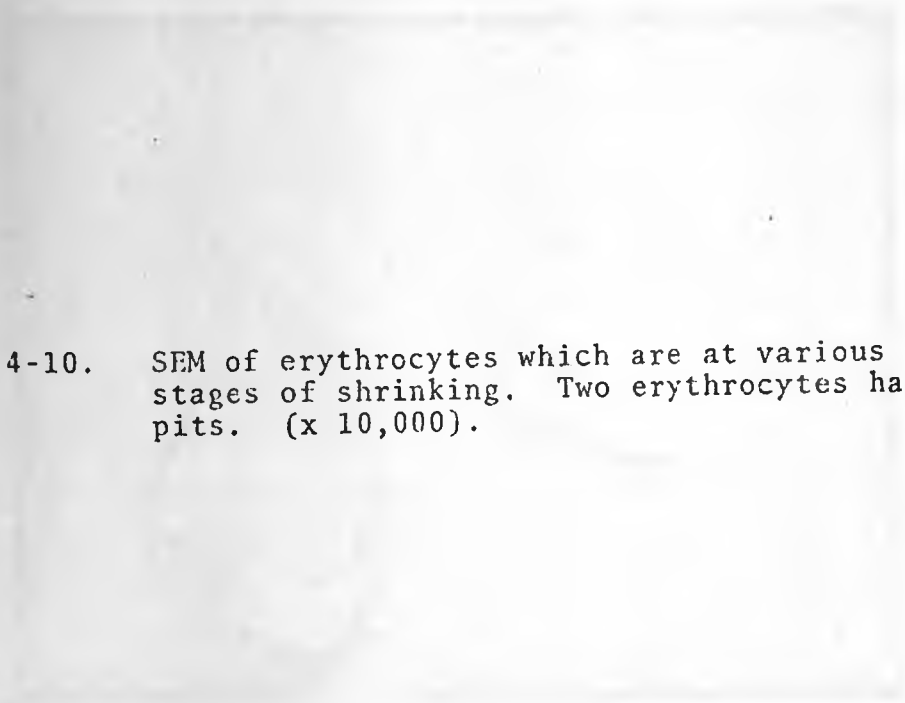
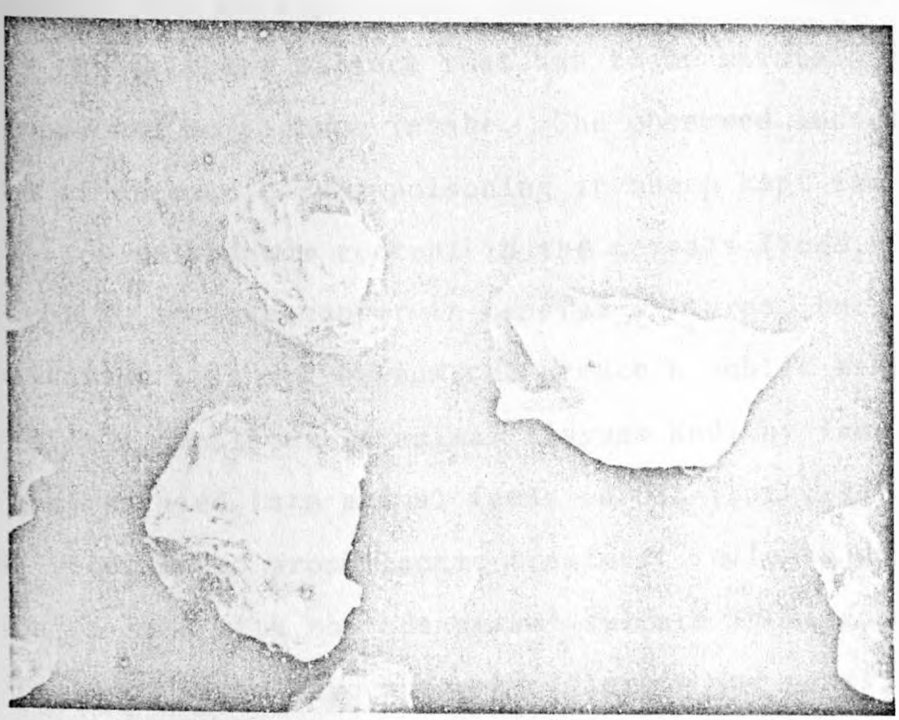


Figure 4-10. SEM of erythrocytes which are at various stages of shrinking. Two erythrocytes have pits. (x 10,000).



CHAPTER V

D-PENICILLAMINE TREATMENT IN CHRONIC COPPER POISONING OF SHEEP

Introduction

Sheep are very susceptible to chronic copper poisoning (Todd, 1969). The main problem, especially with housed sheep, is the delicate balance that has to be maintained between copper and molybdenum intake. The observed increased incidence of chronic copper poisoning in sheep kept indoors is due to low molybdenum content in the cereals (Todd, 1972). It is legal to include copper in mineral mixtures, but supplementation with molybdenum can create a public health hazard as it accumulates in animal tissues and, by law, cannot be incorporated into animal feeds in USA (Buck, 1970).

The recommended prophylactic treatment includes supplementation of food with molybdenum and sulfate in herds where copper toxicity signs have appeared (Pierson and Aanes, 1958; Ross, 1966). This treatment is effective in reducing losses in exposed sheep but does not take care of the few sheep which are already showing toxic signs (Todd, 1962).

Penicillamine has long been known to be effective in treating Wilson's disease (Walshe, 1956; 1966). These patients have a genetic defect in copper metabolism which allows copper accumulation in the liver and other tissues. Long term oral penicillamine treatment reduces copper stores in these patients and eliminates disease symptoms by enhancing copper

excretion.

This study was designed to assess the effect of short term treatment of housed sheep with D-penicillamine on liver copper stores and the effect of the intravenous treatment on various physiological functions.

Materials and Methods

Experimental Procedure

Five housed Southdown sheep, ranging in age from three to five years, were used for this experiment. They had been fed hay all their lives. A liver sample for copper determination was obtained from each sheep by right flank laparotomy or thoracotomy under halothane anesthesia.

After recovery from the surgical wounds, the sheep were put in metabolism cages for at least seven days before urine and fecal collections were commenced. They were fed 1 kg alfalfa pellets daily and tap water ad lib. At the end of the acclimatization period, indwelling urinary foley catheters were placed in the female sheep and a funnel adaptation was made for the male sheep for urine collection. Feces were collected separately into plastic buckets. Total daily collections were made for three days and the collections pooled. Duplicate samples were then taken for copper analysis.

During the control period, heparinized and EDTA venous blood was obtained for plasma and hematology determinations.

At the end of the control period, the sheep were treated with 0.5 g of a 2 percent D-penicillamine solution per day

for 4 consecutive days. The solution was made fresh in isotonic saline and given intravenously in two doses, one in the morning and the rest in the evening. The first day of injections was designated day 0.

Urine and feces were collected daily. The sheep were bled from the jugular vein for plasma determinations and hematology.

The sheep were killed at the end of the treatment period and total liver and kidney weights recorded. Aliquots of these organs were frozen at -20C for future copper analysis.

Analytical Procedures

The heparinized blood was spun at 2000 RPM for 15 minutes in a refrigerated centrifuge.[a] Plasma was harvested and used immediately for analysis or stored at -20C until required.

Copper. Five ml urine samples and a measured amount of feces were freeze dried[b] in duplicate for 24 hours and then dry ashed in a muffle furnace[c] at 500C for 12 hours. Urine ash was dissolved in 2 ml 4 N HCl while the fecal ash was dissolved in 5 ml 4 N HCl, transferred to 25 ml volumetric flasks and brought to volume with deionized distilled water.

Liver samples were processed as described for the feces.

Plasma copper was determined using the method of Olson

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[a] Model J21B, Beckman Instruments, Inc., Fullerton, Cal. 92631.

[b] Model 10-800, Virtis Freeze Drier, Virtis Co., Gardiner, N.Y. 12525.

[c] Hotpack Corp., Philadelphia, Pa. 19135.

and Hamlin (1969). Plasma proteins were precipitated by adding 1 ml of 20 percent trichloroacetic acid (TCA) to 1 ml of plasma, vortexing thoroughly, and treating the mixture at 90C for 15 minutes in a hot water bath. The mixture was cooled and then spun at 2000 RPM for 15 minutes.[a] The supernatant was taken for copper analysis. The dissolved fecal, urine, liver and kidney ashes and the protein-free supernatant from plasma were analyzed for copper by atomic absorption spectrophtometry.[b]

Serum Analysis. Methodologies for plasma GOT, bilirubin, BUN and creatinine are detailed in Chapter II.

Methods for determining hematocrit, hemoglobin, total leukocyte and erythrocyte counts were detailed in Chapter IV of this thesis.

Results

The copper concentration in the liver samples is shown in Table 5-1 and Figure 5-1. The mean (± SEM) of four samples before D-penicillamine treatment was 630 ± 103.9 (398 to 926) ppm dry matter (DM) and mean concentration after treatment was 486 ± 63.6 ppm DM (286 to 631). The decrease of 144 ppm in copper concentration was not statistically significant. Mean apparent total loss due to treatment was 112 mg.

Changes in urinary copper excretion are shown in Figure 5-1 and Table 5-2. The mean urinary copper excretion for the

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[a] Model UV, Damon/IEC Division, Needham Heights, Mass. 02194.

[b] Model 305B, Perkin-Elmer, Norwalk, Conn. 06856.

control period was 0.7 ± 0.2 mg copper (0.33 to 1.41). In 24 hr. D-penicillamine treatment increased excretion to 1.80 ± 0.35 mg copper 24 hours after the first treatment. Peak excretion was reached on the second day of treatment with 2.20 ± 0.23 mg copper per 24 hr in urine. There was a decrease in the amount of copper excreted in urine after the second day. Excretion on the fourth day of treatment was 1.5 ± 0.09 mg/24 hr. Excretion level returned to normal after treatment was stopped on the fourth day. The mean (± SEM) of urinary copper excretion during the four days of treatment was 1.65 ± 0.16 mg/24 hr which was significantly higher ($p < 0.01$) than the control period mean of 0.7 ± 0.2 mg copper per 24 hr. The calculated mean copper loss through the urine over the 4 day treatment period was 3.6 mg which could not account for the observed mean loss of 144 mg from the liver.

Intravenous D-penicillamine treatment did not alter significantly the daily fecal copper excretion as shown in Figure 5-1 and Table 5-2. The mean daily fecal copper excretion in the control period was 7.16 ± 1.97 (± SEM) mg/24 hr as compared to 7.95 ± 1.11 mg/24 hr during the treatment period.

The amount of urine excreted over a 24 hour period was not affected by treatment. Daily urine output before treatment was 837 ± 188 ml (mean ± SEM) (373 to 1383) and the mean output during the treatment period was 943 ± 79.6 ml (380 to 2080). Daily fecal output was not changed by D-penicillamine treatment: 940 ± 214 g before compared with 1135 ± 122

during treatment.

Total plasma copper did not change with D-penicillamine treatment as shown in Figure 5-1. Concentration before D-penicillamine was 1.27 ± 0.04 ug/ml (mean ± SEM) and during the four day treatment it was 1.23 ± 0.04 (Table 5-3). Other plasma parameters analyzed were unaffected by the treatment (Table 5-3). Glutamate oxaloacetate transaminase (GOT) activity was 91 ± 6.3 S/F U/ml (mean ± SEM) before and 72 ± 3.4 S/F U/ml during the 4 days of treatment. Blood urea nitrogen (BUN) and plasma creatinine concentrations were not changed by treatment (Table 5-3).

D-penicillamine administration did not affect the hematology values (Table 5-4). Mean total leukocyte and erythrocyte counts remained normal during the treatment period. The same was observed for PCV and hemoglobin values.

Discussion

The results of this experiment show that D-penicillamine treatment enhanced copper excretion in the urine. The 3.6 mg copper loss through the urine attributable to treatment was infinitely small compared to total hepatic copper of 455 mg. It is unlikely that such a small loss would significantly change the total liver copper. The observed reduction in total hepatic copper was not significant and it can possibly be explained by sampling errors. Barden and Robertson (1962) observed differences in copper concentration of liver samples taken from different sections of the liver. Biopsy and post

mortem liver samples were not necessarily taken from the same liver lobes. This can account for the observed differences in concentrations.

The efficacy of penicillamine in mobilizing copper through the urine has been demonstrated by MacPherson and Hemingway (1967). This drug was superior to ethylenediamine tetraacetic acid (EDTA) and dimercaprol (BAL) in this respect. They gave their sheep only one treatment. Cupriuresis observed in this work lasted only two days. This apparent reduction in penicillamine effectiveness can be explained by depletion of body copper pool (Walshe, 1966). Although most of the sheep had hepatic copper levels above the maximum normal of 400 ppm DM (Underwood, 1977), only one sheep (BF) had exceeded the critical copper concentration of 800 ppm described by Jubb and Kennedy (1970). It is possible that below such critical levels, copper cannot be mobilized with ease from the liver.

There was no change in plasma copper concentration with penicillamine treatment although its mode of action depends upon its ability to render plasma copper more readily available for diffusion across the glomerular membrane (Walshe, 1963). About 80 percent of the plasma copper in sheep is in ceruloplasmin (McCosker, 1968), and it is possible that the copper in this enzyme is not readily extracted by penicillamine (Schubert, 1964). Removal of copper from the "direct" fraction is comparatively small especially when the plasma copper levels are normal as seen here.

Long term oral D-penicillamine is the recommended treatment for Wilson's disease (Walshe, 1966). Such prolonged treatment would be expensive and probably not economically feasible in a sheep operation. It would be interesting to see whether sheep undergoing a hemolytic crisis would be helped by penicillamine treatment. Short term treatment, especially when sheep are not yet toxic but showing abnormal liver function tests (Ishmael et al., 1972), could alter the course of the disease and possibly prevent the precipitation of a hemolytic crisis. The traditional methods for treating chronic copper poisoning depend on supplementing the sheep's diet with 100 mg ammonium molybdate and 1 g sodium sulfate (Ross, 1970). This treatment is effective in prevention but does not alter the course of the disease when signs are incipient (Todd, 1962).

Toxic effects of penicillamine treatment include optic neuritis (Tu et al., 1963) and nephritis syndrome (Sternlieb, 1966). These are attributed to the antipyridoxine action of the L-isomer of penicillamine. There have also been reports of bone marrow depression (Sternlieb and Scheinberg, 1964) after treatment with the D-isomer of penicillamine. Most of these toxic syndromes occur after prolonged treatment. Therefore, the observed normal liver and kidney function tests and hematology parameters were not unexpected. These tests were included to monitor the effects of intravenous D-penicillamine, a treatment which is not usually applied in human medicine.

Summary

Five, three to five year old Southdown sheep had their livers biopsied. They were subsequently treated for four days with 0.5 g D-penicillamine/day given intravenously as a 2 percent solution. The sheep were killed at the end of the treatment period and the liver taken for copper analysis. During the treatment, urine and feces were collected at 24 hr intervals and copper contents were determined. Liver function tests included GOT and bilirubin. Blood urea nitrogen and plasma creatine were measured to evaluate kidney function.

Treatment did not significantly affect the liver copper concentration, fecal copper excretion or plasma copper concentration. Plasma and hematology values remained normal. Treatment was accompanied by increased urinary copper excretion which lasted the whole period of treatment. This cupriuresis was marked during the first two days of treatment. The 3.6 mg copper lost through the urine could not be expected to significantly change hepatic copper concentration.

Table 5-1. Effect of D-penicillamine Treatment on Liver Copper Concentrations of Housed Sheep.

Sheep ID	Sex	Liver Copper Concentration (ug/g DM) Before Penicillamine	After Penicillamine	Difference
10	female	398	406	+6
13	female	600	600	0
16	female	422	286	-136
11	female	804	507	-297
BF	male	926	631	-295
Mean±SEM		630±103.9	486±63.6	-144±66.9

Table 5-2. Urine and Fecal Outputs and Their Copper Content During the D-penicillamine Trial (mean±SEM).

	Control Period	Treatment Period	Significance[a]
Urine output (ml/24 hr)	837.4 ± 188 ($\bar{5}$)[b]	943 ± 79.6 ($\bar{19}$)	ns
Urine copper (mg/24 hr)	0.7 ± 0.2 ($\bar{5}$)	1.65 ± 0.16 ($\bar{19}$)	<0.01
Fecal output (gm/24 hr)	940 ± 214 ($\bar{5}$)	1135 ± 122 ($\bar{19}$)	ns
Fecal copper (mg/24 hr)	7.16 ± 1.97 ($\bar{5}$)	7.96 ± 1.11 ($\bar{19}$)	ns

[a] Student's t test.

[b] Number of observations.

Table 5-3. Plasma Analysis During the D-penicillamine Trial (mean ± SEM).

	Control Period	Treatment Period	Significance[a]
GOT (S/F u/ml)	91 ± 6.3 (15)[b]	72 ± 3.4 (24)	ns
Bilirubin (mg/dl)	0.12 ± 0.016 (14)	0.11 ± 0.001 (24)	ns
BUN (mg/dl)	17.2 ± 1.8 (13)	22.3 ± 1.3 (20)	ns
Creatinine (mg/dl)	0.85 ± 0.023 (14)	0.88 ± 0.04 (25)	ns
Total plasma copper (ug/ml)	1.27 ± 0.04 (14)	1.23 ± 0.04 (26)	ns

[a] Student's t test.

[b] Number of observations.

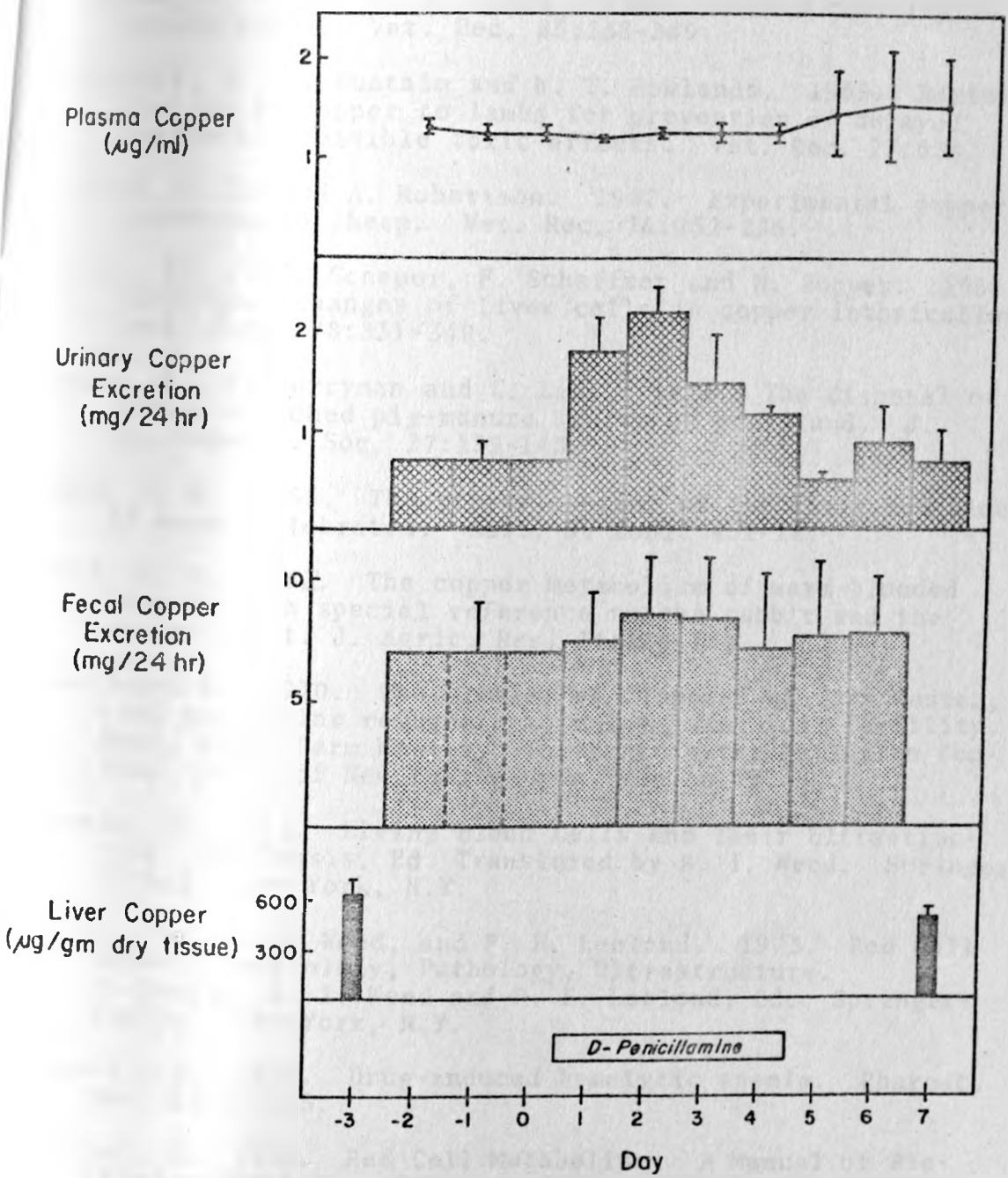
Table 5-4. Summary of Hematology During the D-penicillamine Trial (mean ± SEM).

Parameter	Control Period	Treatment Period	Significance[a]
WBC x 10^3/ul	7.16 ± 0.38 (17)[b]	7.93 ± 0.89 (12)	ns
RBC x 10^6/ul	9.75 ± 0.24 (17)	9.28 ± 0.42 (12)	ns
PCV (%)	33.9 ± 0.89 (17)	32.9 ± 0.98 (20)	ns
Hb (mg/dl)	11.06 ± 0.36	10.13 ± 0.39	ns

[a] Student's t test.

[b] Number of observations.

Figure 5-1. The effect of intravenous D-penicillamine treatment on hepatic copper storage and copper excretion in urine and feces in 5 housed adult sheep given 0.5 gm of 2% D-penicillamine IV daily in two doses. Day 0 represents the time treatment was started. Urine and feces were collected for 3 days before treatment and pooled for copper determination.


Plasma Copper
(µg/ml)
2
1
Urinary Copper
Excretion
(mg/24 hr)
2
1
Fecal Copper
Excretion
(mg/24 hr)
10
5
Liver Copper
(µg/gm dry tissue)
600
300
D-Penicillamine
-3
-2
-1
0
1
2
3
4
5
6
7
Day

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APPENDIX A

Tables for Chapter II.

Table A1. Body Weights of the Sheep in the Chronic Copper Experiment

Copper Poisoned Sheep	Body Weight (kg) Initial	Post-mortem
1450	27.4	25.0
1456	29.3	25.9
371	33.8	26.8
373	28.5	29.5
1473	27.8	23.1
Mean ± SEM	29.3 ± 1.1	26.0 ± 1.0
Significance[a]		$p < 0.05$
1451	31.3	32.2
1454	32.3	31.3
1461	31.4	32.7
Mean ± SEM	31.6 ± 0.3	32.0 ± 0.4
Significance[b]	ns	$p < 0.01$

[a] Difference between post-mortem and initial body weight of copper fed sheep (Student's t test).

[b] Difference in weight of control and copper fed sheep before copper feeding and at post-mortem (Student's t test).

Table A2. Blood Urea Nitrogen (BUN) in Chronic Copper Experiment (mg/dl).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	27.8	23.1	21.1	23.9	21.1	21.1	25.4	20.8
-5	36.0	27.1	23.5	26.9	32.9	48.5	28.1	27.1
0	30.7	28.2	30.5	24.8	37.9	22.4	32.9	32.3
9	20.1	9.9	18.1	17.9	15.8	14.3	15.5	10.0
14	14.1	16.6	14.2	15.3	14.2	12.9	13.6	11.4
21	19.3	11.7	12.1	14.0	14.1	7.8	14.7	6.7
28	6.7	3.4	10.2	5.1	5.8	4.4	4.4	3.5
35	15.5	7.8	10.6	14.6	30.0	12.6	13.2	8.0
42	15.7	11.7	13.8	9.5	10.2	12.5	11.7	9.0
43	-	13.4	-	-	8.3	15.5	-	-
44	-	-	-	-	-	11.2	-	-
46	-	11.3	-	-	-	11.3	-	-
49	9.4	*	12.1	8.2	9.7	7.2	10.4	4.9
54	15.7		62.0	-	15.1	14.1	-	-
55	14.9		-	12.9	13.9	12.1	-	-
56	21.8		-	-	15.0	-	-	7.3
57	17.5		-	-	10.0	-	-	13.6
58	16.8		-	-	7.0	-	-	11.8
63	6.1		9.6	9.9	5.9	9.2	13.8	8.4
70	19.2		12.0	16.7	14.3	15.9	24.9	18.0
77	12.7		24.6	12.7	9.8	8.8	17.2	8.6
84	10.4		10.9	12.5	10.2	11.8	11.0	9.2
91	17.7		24.6	23.0	16.0	16.7	17.2	15.6
92	-		-	-	31.2	22.5	-	-
94	34.3		-	-	*	*	-	-
95	32.8		-	-			-	15.6
98	*		17.9	16.9			19.7	18.6
105			17.0	18.2			20.9	12.3
111			17.1	12.9			19.7	17.4
112			17.3	17.1			20.7	22.8
113			24.7	26.6			23.6	29.2
115			21.8	*			25.0	*
116			47.7				36.1	
117			35.8				36.2	
118			20.4				*	
			*					
Mean ± SEM							16.3 ± 1.04	

* Sheep dead.

Table A3. Plasma Copper in Chronic Copper Experiment (ug/ml).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	0.99	0.83	1.05	0.84	0.94	1.12	0.91	0.93
-5	1.03	0.76	1.02	0.81	0.98	1.12	0.90	0.94
0	1.00	0.81	1.04	0.90	0.98	0.90	0.91	0.95
9	1.17	1.02	1.06	0.95	1.01	0.96	1.15	1.05
14	0.96	1.12	1.01	0.86	0.88	0.75	1.09	0.94
18	-	1.52	-	-	-	0.94	-	-
21	0.87	1.54	0.95	0.82	0.91	0.79	1.06	0.91
28	0.62	0.95	0.68	0.59	0.57	0.54	0.68	0.69
35	0.63	0.86	0.70	0.54	0.54	0.52	0.67	0.68
42	0.70	1.02	0.87	0.67	0.54	0.55	0.67	0.67
43	-	0.88	-	-	0.53	0.55	-	-
44	-	0.86	-	-	-	0.56	-	-
46	-	1.01	-	-	-	0.56	-	-
49	0.84	*	1.13	1.02	0.67	0.88	0.94	0.90
54	1.09		1.39	-	1.20	0.84	-	-
55	1.03		-	0.99	1.13	-	1.00	-
56	0.90		-	-	1.20	-	-	1.12
57	0.98		-	-	1.18	-	-	1.15
58	1.00		-	-	1.46	-	-	1.14
63	1.16		1.27	1.02	1.54	0.88	1.08	1.12
70	0.82		0.91	0.79	1.21	0.66	0.86	0.92
77	0.86		1.00	0.83	1.06	0.68	0.87	0.93
84	0.78		0.86	0.75	0.86	0.67	0.80	0.88
91	0.88		0.78	0.72	3.58	0.61	0.72	0.78
92	-		-	-	1.85	0.70	-	-
94	0.87		-	-	*	*	-	-
95	0.86		-	0.80			-	0.82
98	*		0.82	0.74			0.74	0.82
105			0.88	0.79			0.76	0.83
111			1.21	0.87			0.78	0.83
112			1.18	0.78			0.74	0.84
113			1.15	0.78			0.72	0.80
115			1.31	*			0.76	*
116			1.50				0.80	
117			1.28				0.69	
118			1.34				*	
			*					
Mean ± SEM							0.84 ± 0.02	

* Sheep dead.

Table A4. Creatinine in Chronic Copper Experiment (ug/dl).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	0.90	0.65	0.75	0.65	0.60	0.65	0.80	0.80
-5	1.00	0.70	0.70	0.60	0.55	0.60	0.80	0.85
0	0.95	0.75	0.75	0.60	0.65	0.65	0.85	0.85
9	1.20	0.85	0.85	0.65	0.70	0.75	0.95	0.95
14	1.55	0.90	0.90	0.70	0.75	0.80	1.05	0.90
21	1.30	1.10	0.80	0.80	0.95	0.90	1.20	1.10
28	1.35	1.00	0.80	0.80	0.85	1.05	1.20	1.10
35	1.45	1.00	0.80	0.80	1.00	1.00	1.30	1.10
42	1.40	0.85	0.85	0.60	0.95	1.05	1.30	1.10
43	-	0.75	-	-	0.95	1.10	-	-
44	-	0.80	-	-	-	1.10	-	-
46	-	0.80	-	-	-	1.05	-	-
49	1.25	*	1.05	0.85	1.10	1.05	1.30	1.05
54	1.20		0.95	-	1.05	1.05	-	-
55	1.20		-	0.85	1.00	-	1.30	-
56	1.15		-	-	0.95	-	-	0.95
57	1.05		-	-	0.95	-	-	0.95
58	0.95		-	-	0.75	-	-	0.85
63	1.00		0.75	0.70	0.70	0.90	1.20	0.80
70	1.05		0.75	0.70	0.70	0.80	1.10	0.85
77	1.05		0.75	0.65	0.70	0.80	1.05	0.85
84	0.90		0.70	0.70	0.80	0.90	1.05	0.90
91	1.00		0.80	0.70	1.00	0.80	1.10	0.90
92	-		-	-	1.15	0.90	-	-
94	1.00		-	-	*	*	-	-
95	1.10		-	0.70			0.90	-
98	*		0.80	0.65			1.05	0.95
105			0.90	0.70			1.10	1.00
111			0.85	0.70			1.15	1.00
112			0.85	0.70			1.00	0.95
113			0.80	0.75			1.00	0.95
115			0.70	*			0.80	*
116			0.80				0.85	*
117			0.70				*	
			*					
Mean ± SEM						0.97 ± 0.02		
								0.84 ± 0.02

* Sheep dead.

Table A5. Alkaline Phosphatase (AP) in Chronic Copper Experiment (SU/ml).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	3.0	3.8	2.9	4.1	4.6	2.4	3.9	4.0
-5	3.0	3.6	3.2	4.7	4.6	2.7	4.3	4.3
0	2.9	3.5	3.4	4.3	4.8	3.6	5.8	5.0
9	2.2	2.1	3.1	3.9	4.7	3.1	4.3	3.7
14	2.1	5.5	2.6	4.0	4.0	2.8	4.9	3.2
18	1.7	3.5	2.2	3.4	3.3	1.9	3.5	2.0
21	1.7	3.2	1.7	3.4	3.0	2.1	3.3	2.2
28	1.9	2.8	1.5	2.3	2.6	1.7	2.6	1.7
35	1.6	3.0	1.4	2.4	2.3	2.05	3.15	1.65
42	1.7	1.8	1.0	1.5	2.0	1.9	2.4	1.7
43	-	2.9	-	-	2.3	1.8	-	-
44	-	2.3	-	-	-	1.5	-	-
46	-	2.5	-	-	-	1.55	-	-
49	1.9	*	1.35	1.55	2.7	1.9	2.4	2.0
54	3.5		0.9	-	3.3	1.3	-	-
55	1.9		-	1.4	2.0	-	1.8	-
56	1.2		-	-	1.6	-	1.3	-
57	1.0		-	-	1.2	-	1.2	-
58	1.3		-	-	1.3	-	1.2	-
63	0.9		1.2	1.3	1.8	1.5	1.9	1.3
70	1.4		1.1	1.9	1.2	1.6	2.5	1.5
77	2.1		1.6	1.8	2.4	2.0	2.3	1.6
84	1.8		1.4	1.7	3.2	1.7	2.0	1.3
91	2.2		1.3	1.4	2.2	1.3	2.3	1.2
92	-		-	-	0.4	1.7	-	-
94	5.4		-	-	*	*	-	-
95	2.7		-	1.8			-	1.4
98	*		1.6	1.9			2.8	1.8
105			1.5	2.0			2.4	1.5
111			-	-			-	-
112			-	-			-	-
113			1.4	1.6			1.6	1.2
115			1.3	*			1.8	*
116			2.3				2.1	
117			1.3				2.3	
			*				*	
Mean ± SEM						2.3 ± 0.13		

* Sheep dead.

Table A6. Total Plasma Bilirubin in Chronic Copper Experiment (mg/dl).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	0.1	0.1	0.1	0.1	0.1	0.1	0.05	0.1
-5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.05
0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
9	0.1	0.1	0.1	0.1	0.05	0.1	0.1	0.05
14	0.15	1.0	0.15	0.15	0.1	0.15	0.15	0.15
18	0.15	1.2	0.2	0.1	0.2	0.2	0.2	0.15
21	0.2	2.05	0.15	0.25	0.2	0.2	0.2	0.15
28	0.2	0.7	0.1	0.15	0.15	0.1	0.15	0.1
35	0.15	0.3	0.1	0.15	0.1	0.1	0.15	0.1
42	0.3	2.3	0.1	0.1	0.15	0.1	0.1	0.1
43	-	2.25	-	-	0.2	0.1	-	-
44	-	1.55	-	-	-	0.1	-	-
46	-	1.25	-	-	-	0.15	-	-
49	0.2	*	0.15	0.2	0.15	0.1	0.15	0.1
54	0.2		0.05	-	1.3	0.05	-	-
55	0.3		-	0.2	1.25	-	0.15	-
56	0.2		-	-	1.0	-	-	0.05
57	0.6		-	-	0.9	-	-	0.15
58	0.6		-	-	0.65	-	-	0.1
63	0.3		0.1	0.15	0.3	0.1	0.15	0.15
70	0.15		0.05	0.1	0.05	0.1	0.1	0.1
77	0.1		0.1	0.1	0.1	0.1	0.1	0.1
84	0.05		0.05	0.05	0.05	0.1	0.1	0.05
91	0.15		0.1	0.05	0.85	0.1	0.05	0.05
92	-		-	-	2.1	0.05	-	-
94	0.5		-	-	*	*	-	-
95	0.7		-	0.05			-	-
98	*		0.1	0.1			0.1	0.1
105			0.1	0.1			0.1	0.1
111			0.3	0.1			0.1	0.1
112			0.6	0.2			0.1	0.1
113			0.35	0.1			0.15	0.05
115			0.2	*			0.05	*
116			0.75				0.05	
117			1.35				0.1	
118			0.3				*	
			*					
Mean ± SEM						0.11 ± .004		

* Sheep dead.

Table A7. Glutamate Oxaloacetate Transaminase (GOT) in Chronic Copper Experiment (S/F U/ml).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	69	58	50	64	74	59	57	58
-5	58	53	35	45	56	48	43	47
0	40	49	43	47	56	54	49	55
9	51	97	36	41	81	42	39	47
14	53	216	47	45	68	60	50	49
18	55	1040	48	45	78	65	62	50
21	56	1170	42	45	90	61	56	52
28	48	600	40	43	59	52	52	50
35	42	130	36	41	53	48	45	42
42	82	290	43	40	72	51	53	49
43	-	390	-	-	76	51	-	-
44	-	460	-	-	-	46	-	-
46	-	400	-	-	-	45	-	-
49	55	*	45	44	81	55	52	47
54	190		-	-	450	47	-	41
55	160		-	-	290	43	-	42
56	110		-	-	260	-	-	39
57	256		-	-	210	-	-	55
58	99		-	-	158	-	-	53
63	81		31	47	94	52	56	41
70	59		41	45	130	49	48	53
77	103		57	50	161	65	72	69
84	92		57	43	125	88	78	64
91	133		87	54	660	69	76	67
92	-		-	-	620	61	-	-
94	285		-	-	*	*	-	-
95	255		-	64			-	-
98	*		74	68			75	73
105			73	64			74	64
111			124	75			74	63
112			69	69			35	54
113			151	81			64	59
115			460	*			79	*
116			590				85	
117			580				76	
118			200				*	
			*					
Mean ± SEM						56.3 ± 1.4		

* Sheep dead.

Table A8. Plasma Ceruloplasmin in Chronic Copper Experiment (IU/L).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	39.8	29.0	43.6	39.8	36.6	44.7	34.5	37.7
-5	42.9	29.0	44.3	29.3	40.7	46.8	34.6	43.6
0	32.1	23.2	37.3	29.9	30.9	29.5	27.9	35.1
9	34.2	26.2	32.8	26.2	28.3	26.5	42.6	31.8
14	36.3	31.4	39.1	26.9	29.3	39.4	45.0	41.5
18	38.0	39.8	41.2	28.3	30.0	32.1	44.0	38.4
21	30.0	30.7	34.9	29.0	27.2	23.7	39.1	34.6
28	25.1	27.9	29.7	22.3	20.2	21.3	29.7	32.8
35	21.3	27.2	26.5	18.1	17.1	20.9	35.6	37.7
42	21.6	17.5	40.8	31.4	24.3	23.7	31.1	28.3
43	-	17.8	-	-	28.3	23.0	-	-
44	-	17.8	-	-	-	21.6	-	-
46	-	11.5	-	-	-	23.0	-	-
49	18.5	*	37.3	33.9	23.6	30.0	30.4	30.4
54	19.5		48.9	-	20.2	25.1	-	-
55	26.2		30.0	-	29.3	-	31.4	-
56	28.1		-	-	27.6	-	-	35.6
57	22.3		-	-	29.0	-	-	21.9
58	28.3		-	-	28.9	-	-	24.7
63	27.2		21.9	27.9	25.3	25.0	34.9	20.1
70	28.1		18.5	17.8	20.6	22.5	21.3	20.6
77	21.5		22.0	17.5	29.2	25.0	21.6	25.5
84	22.2		20.2	16.4	25.4	24.7	19.9	23.7
91	27.3		25.7	24.0	28.5	21.3	25.0	20.9
92	-		-	-	9.4	24.7	-	-
94	26.2		-	-	*	*	-	-
95	15.0		-	26.1			-	22.3
98	*		24.3	23.6			16.8	21.6
105			16.8	14.3			16.4	22.0
111			19.9	22.6			14.7	19.5
112			21.6	22.6			15.7	20.5
113			22.3	23.6			18.2	23.7
115			38.0	*			20.6	*
116			33.5				21.6	
117			26.2				17.1	
118			32.5				*	
			*					
Mean ± SEM						27.9 ± 0.97		

* Sheep dead.

Table A9. Ceruloplasmin Assay. Effect of Hemoglobin on Choice of Blank.

Sheep ID	Days After Crisis	Ceruloplasmin (IU/L) Reagent Blank	Plasma Blank
1450*	0	36.3	19.5
	0	34.6	26.2
	1	34.6	26.2
	1	49.2	15.0
1456	0	23.7	17.5
	2	30.7	17.8
	3	28.6	17.8
	4	24.1	11.5
371	0	40.1	38.0
	1	40.8	33.5
1473*	0	43.6	20.2
	0	98.0	9.4
	1	38.0	29.3
Mean ± SEM		44.9 ± 6.7	20.2 ± 2.6
p (Student's t test)		<0.01	

* These two sheep had two hemolytic crises.

Table A10. Total Bilirubin in Subacute Experiment (mg/dl).

Day	Copper Fed Sheep		Control Sheep	
	116	133	114	129
-10	.1	.1	.1	.1
-9	.1	.1	.05	.05
-8	.1	.05	.1	.1
-2	.1	.1	.1	.1
0	.25	.1	.1	.05
3	.1	.1	.1	.1
4	.1	.4	.1	.1
6	-	.1	-	-
7	.1	.15	.1	.1
8	.05	-	-	-
10	.1	-	-	-
11	.15	.15	.15	.1
14	.15	.15	.15	.1
18	.1	.25	.1	.1
21	3.35	1.7	.05	.1
24	-	4.6	-	-
25	3.7	4.3	.1	.1
26	-	3.15	.05	-
27	.25		-	-
28	.2		.1	.1
32	-		-	-
35	.15		.15	.1
42	.2			.1

Table A11. GOT in Subacute Experiment (S/F U/ml).

Day	Copper Fed Sheep		Control Sheep	
	116	133	114	129
-10	45	38	38	61
-9	45	49	55	63
-8	49	63	54	44
-2	54	56	46	60
0	50	54	40	56
3	45	175	49	66
4	12	940	46	68
6	-	670	-	-
7	64	316	68	66
8	256	-	-	-
10	220	-	-	-
11	186	275	86	82
14	149	335	70	98
18	129	1740	97	91
21	2100	2070	64	73
25	2400	1660	74	98
26	-	1320	79	-
27	1320		-	-
28	960		82	100
32	456		40	57
35	240		44	58
42	380			55

Table A12. Plasma Copper in Subacute Experiment (mg/ml).

Day	Copper Fed Sheep		Control Sheep	
	116	133	114	129
-10	.75	.73	.70	.80
-9	.76	.72	.76	.89
-8	.79	.80	.78	.83
-2	.92	.72	.87	.91
0	.84	.72	.88	.94
3	.99	.88	.85	1.01
4	1.18	1.50	.82	.95
7	1.25	1.22	.89	.97
8	1.88	-	-	-
10	1.34	-	-	-
11	1.71	1.25	.73	1.00
14	1.46	1.26	.69	.96
17	-	-	.72	1.00
18	1.31	2.07	-	1.02
21	2.26	2.09	1.60	1.03
25	2.08	2.38	1.66	1.06
27	1.32	1.62	1.42	-
28	1.12		1.41	.91
32	1.24		1.24	.98
35	1.42		1.19	.98
42	1.53			1.04

Table A13. Urinary Copper Excretion in Subacute Experiment (ug/24 hr).

Day	Copper Fed Sheep		Control Sheep	
	116	133	114	129
-10	31.39	25.96	28.67	26.97
-9	74.55	42.56	54.15	47.32
-8	67.83	39.30	65.88	65.60
0	164.82	50.70	62.10	96.53
4	329.90	1042.60	129.48	64.86
7	384.75	28.80	46.20	22.00
8	954.00	-	-	-
9	2678.00	-	-	-
10	503.90	-	-	-
11	170.15	80.66	35.67	22.62
14	114.00	149.58	27.62	48.09
17	-	-	-	-
18	842.40	744.60	29.89	53.94
21	3936.80	1683.00	-	60.84
25	5670.00	864.00	-	
27	-		-	
28	521.64		37.52	
32	285.65			
35	426.53			
42	890.65			

Table A14. Fecal Copper Excretion in Subacute Experiment (mg/24 hr).

Day	Copper Fed Sheep		Control Sheep	
	116	133	114	129
-10	6.99	5.41	7.39	6.91
-9	8.67	10.71	8.76	11.96
-8	10.62	11.08	9.91	9.45
0	10.78	6.61	15.47	7.87
4	319.21	63.44	9.66	9.74
7	300.06	-	10.51	9.92
8	-	26.21	-	-
9	-	10.45	-	-
10	-	10.56	-	-
11	49.49	83.68	7.73	10.32
14	12.99	310.00	10.71	11.36
17	-		-	-
18	41.47		8.94	13.66
21	208.85		11.53	11.26
25	270.22		-	-
27	-		-	-
28	45.87		9.27	27.96
32	11.83		7.78	9.31
35	11.30		10.31	10.06
42	18.83			17.03

APPENDIX B

Tables for Chapter III.

Table B1. Subcellular Copper Distribution in Chronic Study (ug Copper/mg Protein).

	Fraction			
	P_1	P_2	P_3	S_3
Copper Poisoned Sheep				
1450	8.6	18.5	2.3	2.8
1456	6.9	1.7	0.4	1.2
371	61.6	8.2	1.0	2.7
1473	38.8	15.2	1.3	5.0
Mean	29.0	10.9	1.2	2.9
± SEM	± 13.1	± 3.7	± 0.4	± 0.8
Control Sheep				
1451	1.6	1.8	0.21	0.89
1454	3.8	1.6	0.47	0.75
1461	2.1	2.1	0.09	0.32
Mean	2.5	1.8	0.26	0.65
± SEM	± .67	± .15	± .11	± .17
Significance (Student's t test)	ns	ns	ns	ns

Table B2. Liver Acid Phosphatase in Chronic Study.

	Fractions				
	H_1	P_1	P_2	P_3	S_3
	SU/g liver		Percent of H_1		
Copper Poisoned Sheep					
1450	245	45.0	8.0	9.7	21.7
1456	204	37.6	13.5	6.7	23.4
371	173	57.7	30.0	5.8	16.8
1473	216	37.3	5.7	5.9	9.9
Mean	209	44.4	14.3	7.0	17.9
± SEM	± 15	± 4.7	± 5.4	± 0.9	± 3.0
Control Sheep					
1451	249	20.8	22.1	10.3	21.1
1454	161	60.3	16.3	10.2	31.5
1461	172	25.9	20.7	-	25.8
Mean	194	35.6	19.7	10.2	26.1
± SEM	± 28	± 12.4	± 1.7	± .05	± 3.0

Table B3. Subcellular Copper Distribution. Percent of Homogenate Copper in Each Fraction.

	Fraction			
	P_1	P_2	P_3	S_3
Copper Poisoned Sheep				
1450	43.4	35.2	5.0	11.1
1456	45.8	13.9	2.2	17.4
371	45.6	31.5	7.3	13.5
1473	42.0	9.0	3.7	26.8
Mean	44.2	22.4	4.5	17.0
± SEM	± 0.91	± 6.50	± 1.08	± 3.78
Control Sheep				
1451	17.3	43.1	5.0	26.4
1454	25.0	26.1	17.7	19.3
1461	25.7	43.0	3.4	16.1
Mean	22.7	37.4	8.7	20.6
± SEM	± 2.69	± 5.65	± 4.52	± 2.18
Significance (Student's t test)	$p < 0.01$	ns	ns	ns

Table B4. Subcellular Protein Distribution (mg/g Fresh Liver).

	Fraction					
	H1	P_1	P_2	P_3	S_3	Recovery (%)
Copper Poisoned Sheep						
1450	178.5	75.9	26.8	32.5	60.3	92.8
1456	196.0	39.2	49.3	35.0	83.8	105.0
371	178.0	6.4	33.2	65.0	44.1	83.3
1473	144.5	15.2	8.3	41.6	75.8	97.0
Mean	139.4	34.2	29.4	43.5	66.0	94.5
± SEM	± 35.8	± 15.5	± 10.3	± 7.4	± 8.8	± 9.0
Control Sheep						
1451	187.0	21.5	48.1	46.3	57.9	92.8
1454	185.0	13.2	32.3	75.4	51.8	93.0
1461	224.0	21.4	37.5	62.0	88.0	93.0
Mean	198.7	18.7	39.3	61.2	65.9	92.9
± SEM	± 12.7	± 2.8	± 4.6	± 8.4	± 11.1	± 0.1
Significance (Student's t test)	ns	ns	ns	ns	ns	ns

APPENDIX C

Tables for Chapter IV.

Table C1. PCV (%).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	38.5	38.5	35.0	37.5	37.5	35.5	36.5	37.0
-5	39.0	34.5	35.0	37.5	37.0	34.5	36.5	37.5
0	38.5	32.5	35.0	36.0	34.5	36.0	37.0	36.5
9	40.5	33.5	36.5	37.0	33.5	34.0	37.0	36.5
14	39.0	35.0	32.5	37.0	32.5	34.0	35.0	33.5
18	41.0	32.5	38.0	34.5	35.5	35.0	39.5	35.2
21	38.5	31.0	32.5	37.5	31.5	33.0	35.0	34.5
28	35.5	26.0	31.5	35.5	28.0	32.0	36.0	33.5
35	42.0	29.0	33.5	34.0	31.0	34.0	35.5	35.0
39	-	29.0	-	-	29.0	34.0	-	-
42	37.0	22.0	29.5	33.5	28.5	32.5	34.5	34.5
43	-	17.5	-	-	28.5	32.5	-	-
44	-	13.6	-	-	-	27.5	-	-
46	-	12.0	-	-	-	30.5	-	-
49	32.0	*	33.0	35.5	29.0	27.0	33.0	31.0
54	28.0		28.0	-	23.0	30.5	-	-
55	30.5		-	32.0	18.5	-	32.0	-
56	21.0		-	-	17.5	-	-	33.5
57	21.5		-	-	16.0	-	-	30.5
58	24.0		-	-	18.5	-	-	31.5
63	28.5		27.0	30.0	17.5	29.0	33.0	29.0
70	35.0		24.0	30.0	26.5	32.0	36.0	33.5
77	37.0		30.0	29.0	30.5	31.0	32.0	29.5
84	38.0		26.0	29.0	32.5	32.5	30.5	29.0
91	36.0		28.0	28.0	34.0	30.0	32.0	28.0
92	-		-	-	23.0	33.0	-	-
94	9.0		-	-	*	*	-	-
95	6.5		-	34.0			-	32.0
97	11.5		-	-			-	-
98	*		22.0	25.0			31.0	27.5
105			24.0	30.5			30.0	26.5
111			24.0	32.0			28.5	27.0
112			23.0	31.0			30.0	28.0
115			14.0	-			27.0	-
116			12.0	-			31.0	-
119			*	28.5			*	29.5
126				37.0				32.5
Mean ± SEM						32.5 ± 0.36		

* Sheep dead.

Table C2. Hemoglobin (g/dl).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	12.4	13.4	12.4	12.8	13.3	11.7	11.8	14.5
-5	13.3	12.3	11.5	11.5	12.3	12.5	12.5	12.6
0	12.5	10.3	12.0	11.8	12.0	12.4	12.3	12.3
9	12.6	10.2	11.9	12.1	11.8	12.5	12.5	12.5
14	12.5	12.1	10.3	12.0	10.8	11.3	11.3	11.5
18	14.5	10.0	-	-	-	11.8	12.6	12.8
21	12.6	10.8	13.0	13.2	12.0	11.5	11.8	12.0
28	13.0	9.3	10.1	12.0	10.8	11.5	12.5	12.0
35	14.4	9.8	11.8	12.0	10.5	11.3	12.3	12.9
39	-	11.2	11.3	10.6	10.6	13.6	-	-
42	12.6	7.5	-	-	11.3	11.8	12.3	12.0
43	-	5.8	10.5	10.8	10.6	10.1	-	-
44	-	4.3	-	-	10.0	9.8	-	-
46	-	3.5	-	-	-	10.3	-	-
49	10.8	*	11.5	-	-	10.0	11.5	11.0
54	9.3		9.3	11.0	10.3	10.5	-	-
55	9.8		-	-	7.8	-	9.8	-
56	6.9		-	8.3	5.3	-	-	12.0
57	5.3		-	-	5.8	-	-	8.8
58	6.5		-	-	4.8	-	-	8.5
63	8.8		9.5	-	5.0	10.5	10.3	8.8
70	9.5		7.3	9.8	5.3	10.0	10.8	9.6
77	10.3		8.8	7.9	7.8	10.5	9.3	8.8
84	11.2		8.0	8.5	9.2	9.7	8.8	8.5
91	10.0		8.6	8.0	9.3	9.3	9.9	10.3
92	-		-	8.0	10.3	10.0	-	-
94	4.1		-	-	9.7	*	-	-
95	2.9		-	-	*		-	9.8
98	*		6.3	9.8			-	8.2
105			7.3	6.5			8.8	8.4
111			7.5	9.1			9.5	8.6
112			7.8	7.8			9.1	7.5
115			4.2	9.7			7.9	-
116			3.5	-			7.8	-
119			*	-			10.0	9.8
126				8.9			*	10.8
				*				*
Mean ± SEM						10.7 ± 0.19		

* Sheep dead.

Table C3. Total Plasma Protein (g/dl).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	6.5	6.0	6.1	6.0	6.3	6.7	5.8	6.4
-5	6.5	5.8	6.1	6.2	6.2	6.2	5.9	6.6
0	6.5	6.2	6.4	6.3	6.2	5.9	6.0	6.8
9	7.4	6.4	6.5	6.3	6.7	6.5	6.4	6.9
14	6.6	6.4	6.1	6.1	6.1	5.8	6.2	6.7
18	7.3	7.2	7.0	6.8	7.4	6.8	7.0	7.3
21	6.6	6.9	6.1	6.5	6.7	5.8	6.5	6.8
28	6.2	6.7	5.9	6.2	6.3	5.8	6.2	6.8
35	6.6	6.5	6.0	6.4	6.2	5.7	6.1	6.9
42	6.2	6.1	5.4	5.9	5.7	6.0	6.0	6.9
43	-	5.9	-	-	5.8	6.2	-	-
44	-	5.9	-	-	-	5.9	-	-
46	-	6.0	-	-	-	5.7	-	-
49	6.0	5.7	5.4	6.2	5.6	5.8	5.8	6.7
54	5.7	*	5.3	-	5.0	5.5	-	-
55	5.8		-	5.8	4.8	-	5.6	-
56	5.1		-	-	5.2	-	-	5.9
57	5.8		-	-	5.3	-	-	6.8
58	5.5		-	-	5.4	-	-	6.0
63	6.2		5.4	6.1	6.1	5.5	5.6	6.4
70	6.2		5.6	6.1	6.5	5.8	5.9	6.1
77	6.0		5.2	5.9	6.0	5.4	5.5	6.4
84	6.1		5.1	5.8	5.9	5.6	5.5	6.4
91	5.8		4.7	5.8	6.6	5.3	5.5	6.4
92	-		-	-	7.6	6.1	-	-
94	6.8		-	-	*	*	-	-
95	6.0		-	6.5			-	6.8
97	5.6		-	-			-	-
98	*		4.4	5.6			5.4	6.4
105			4.5	5.6			5.3	6.2
111			4.9	5.9			5.1	6.1
112			4.6	5.5			5.2	6.1
115			4.5	-			5.0	-
116			4.7	-			5.3	-
119			*	5.7			*	6.3
				*				*
Mean ± SEM						6.1 ± 0.06		

* Sheep dead

Table C4. Reduced Glutathione (GSH) (mg/100 ml RBC).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	54.8	58.0	87.6	83.8	87.7	63.3	74.0	70.6
-5	89.9	95.5	89.4	86.4	87.6	94.9	103.9	71.3
0	96.3	111.7	105.8	111.2	107.1	99.9	106.5	78.3
9	86.6	102.4	101.4	100.8	106.1	92.8	98.2	89.4
14	74.5	80.4	76.5	77.2	73.7	67.0	77.0	62.8
20	69.5	66.5	72.6	69.1	61.2	68.4	84.8	69.6
28	62.2	73.9	74.5	67.1	72.3	66.1	76.2	50.2
35	65.4	48.4	56.3	53.2	46.2	70.7	61.8	60.1
39	-	41.2	-	-	52.3	54.0	-	-
42	58.1	54.0	72.2	73.4	66.7	69.6	76.5	73.3
43	-	51.8	-	-	56.0	60.1	-	-
44	-	42.1	-	-	-	51.8	-	-
46	-	67.5	-	-	-	46.0	-	-
54	34.4	*	48.4	-	38.0	58.4	-	-
55	-		-	-	-	-	-	-
56	48.9		-	-	60.3	-	-	-
57	-		-	-	-	-	-	-
58	-		-	-	-	-	-	-
63	53.7		51.7	53.3	63.0	50.6	64.1	47.9
70	33.9		60.8	60.0	57.2	52.8	53.9	49.4
77	26.2		53.1	51.5	64.8	49.3	61.5	56.3
84	27.8		57.0	51.5	67.5	55.7	58.4	49.1
91	30.3		54.2	57.9	11.5	43.3	50.2	46.9
92	-		-	-	16.7	65.4	-	47.1
94	66.0		-	-	*	*	-	50.3
95	112.8		-	40.7			-	52.2
98	*		54.6	55.3			68.7	49.9
105			50.1	49.2			60.8	-
112			54.4	51.8			66.3	-
115			35.9	-			44.5	45.1
116			78.0	-			40.1	43.4
119			*	52.0			*	*
				*				
Mean ± SEM						64.0 ± 2.13		

* Sheep dead.

Table C5. Gluthatione Reductase (GR) (IU/g Hb).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	3.39	2.36	2.25	2.50	2.57	3.03	3.57	2.68
-5	2.94	2.74	2.21	3.04	2.32	2.94	2.16	2.74
0	3.70	3.47	4.35	4.50	4.47	3.22	3.09	3.94
9	2.05	2.60	1.85	2.11	1.95	1.55	2.39	1.50
14	1.88	2.36	1.31	1.42	1.38	1.61	1.40	1.75
21	2.32	3.73	1.56	1.88	1.53	2.88	2.11	1.66
28	1.79	2.72	1.29	1.95	1.23	1.32	1.34	1.45
35	2.14	3.03	1.69	1.86	2.02	1.70	1.30	0.77
42	2.02	3.42	1.75	2.81	2.01	1.61	1.45	1.61
43	-	2.92	-	-	2.32	1.46	-	-
44	-	4.22	-	-	-	1.61	-	-
46	-	9.28	-	-	-	1.36	-	-
49	2.44	*	1.70	1.80	1.56	1.31	2.14	1.80
54	3.70		2.57	-	3.05	2.14	-	-
55	1.86		-	2.06	2.32	-	1.69	-
56	1.84		-	-	3.04	-	2.30	-
57	1.99		-	-	2.44	-	1.80	-
58	3.09		-	-	3.26	-	2.14	-
63	4.15		1.41	1.61	5.40	1.46	1.73	2.10
70	5.83		2.11	2.60	5.90	2.09	2.41	2.52
77	4.42		2.62	1.98	7.42	1.66	1.88	2.64
84	3.36		3.82	3.59	3.45	1.45	1.46	1.88
91	3.63		2.28	3.62	3.66	2.94	2.89	2.63
92	-		-	-	1.97	1.85	-	-
94	6.78		-	-	*	*	-	-
95	6.70		-	2.05			-	2.58
98	*		2.54	2.09			2.22	2.14
105			3.86	4.07			2.30	3.20
112			3.93	3.39			2.97	3.05
115			2.30	-			2.85	-
116			6.43	-			3.59	-
119			*	3.89			*	2.34
				*				*
Mean ± SEM						2.13 ± 0.088		

* Sheep dead.

Table C6. Glucose-6-Phosphate Dehydrogenase (G6PD) (IU/g Hb).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	0.52	0.95	0.88	0.77	1.18	0.67	1.10	0.83
-5	1.5	1.15	1.56	1.35	2.23	0.64	1.00	1.61
0	1.06	1.08	1.29	1.00	1.29	1.71	1.04	1.37
9	1.07	1.30	1.11	1.05	0.95	0.76	0.92	1.05
14	0.91	0.66	0.84	0.67	1.09	0.68	0.73	0.63
21	1.00	1.34	1.00	1.07	1.29	0.75	1.07	0.95
28	0.89	1.10	0.77	0.71	1.04	0.61	0.70	0.75
35	1.17	1.57	1.05	1.14	1.88	0.56	1.09	0.96
42	0.96	0.80	0.79	0.91	1.25	0.92	0.68	0.72
43	-	1.13	-	-	1.45	0.79	-	-
44	-	1.21	-	-	-	0.72	-	-
46	-	3.22	-	-	-	1.09	-	-
49	1.17	*	0.71	0.80	1.40	0.69	0.93	0.80
54	1.33		1.82	-	1.48	1.17	-	-
55	0.64		-	0.79	0.88	-	0.61	-
56	1.00		-	-	1.71	-	-	1.00
57	0.91		-	-	1.34	-	-	0.80
58	1.33		-	-	1.33	-	-	0.80
63	2.14		0.96	1.00	4.74	0.56	0.90	1.16
70	2.01		1.38	1.25	2.74	0.80	1.14	1.06
77	1.73		1.33	1.57	3.57	0.88	0.73	1.07
84	1.53		0.69	1.13	0.97	0.98	0.69	0.68
91	1.62		1.23	1.12	1.24	0.67	1.55	0.87
92	-		-	-	1.18	2.06	-	-
94	1.72		-	-	*	*	-	-
95	5.36		-	0.97			-	1.42
98	*		1.53	1.49			1.53	1.09
105			0.97	1.46			1.15	1.02
112			1.66	1.59			1.03	1.05
115			3.23	*			1.06	*
116			3.86				0.84	
			*				*	
Mean ± SEM						0.95 ± 0.038		

* Sheep dead.

Table C7. Glutathione Peroxidase (GSH-Px) (IU/g Hb).

	Copper Fed Sheep					Control Sheep		
Day	1450	1456	371	373	1473	1451	1454	1461
-8	20.6	14.7	27.1	20.5	26.8	35.3	10.8	15.7
-5	23.2	18.0	25.7	25.9	27.1	31.2	22.3	30.4
0	15.3	21.3	14.2	12.9	17.9	15.1	9.3	10.2
9	23.2	23.4	20.7	33.0	27.1	29.2	23.0	21.3
14	13.7	15.3	13.7	12.4	13.8	15.2	15.3	10.7
21	12.6	15.3	19.0	24.1	21.4	18.1	19.0	11.8
28	18.4	28.7	17.0	20.4	25.8	25.1	28.1	23.1
35	13.4	19.6	17.0	21.5	21.1	15.1	17.0	14.5
42	14.6	18.0	22.2	22.4	16.6	21.6	17.0	13.1
43	-	13.4	-	-	14.0	16.1	-	-
44	-	20.5	-	-	-	20.8	-	-
46	-	15.4	-	-	-	10.5	-	-
49	15.6	*	20.9	21.9	19.9	22.4	28.8	21.4
54	19.9		35.4	-	47.9	19.0	-	-
55	14.5		-	23.2	28.4	-	17.4	-
56	19.7		-	-	23.5	-	-	14.8
57	17.6		-	-	21.5	-	-	24.4
58	21.2		-	-	22.3	-	-	26.9
63	4.7		18.8	20.6	9.0	13.3	19.2	16.6
70	7.2		29.6	27.6	12.4	25.7	31.0	28.6
77	6.7		22.4	30.0	12.8	22.5	27.4	17.1
84	5.6		17.8	18.2	7.7	17.3	15.4	11.3
91	12.5		46.8	49.9	47.0	47.1	46.3	36.6
92	-		-	-	24.6	-	-	-
94	17.5		-	-	*	*	-	-
95	3.1		-	10.9			-	10.9
98	*		31.8	37.5			30.4	24.6
105			14.6	22.1			19.9	26.1
112			28.3	33.8			36.1	24.5
116			22.1	-			23.7	-
119			*	11.0			*	12.2
126				13.4				12.8
				*				*
Mean ± SEM						20.1 ± 0.89		

*Sheep dead.

APPENDIX D

Tables for Chapter V.

Table D1. Effect of Penicillamine Treatment on Total Liver Copper of Housed Sheep.

Sheep ID	Total Liver Copper (mg) Before Penicillamine	After Penicillamine	Difference
10	281	287	+6
13	339	339	0
16	273	185	-88
11	708	447	-261
BF	676	461	-215
Mean ± SEM	455 ± 97.4	343 ± 51.4	-112 ± 54.7

Table D2. Plasma Copper (ug/ml).

Day	Sheep ID				
	BF	11	13	10	16
-2	0.98	1.21	1.40	1.44	1.41
-1	1.02	1.16	1.38	1.35	1.38
0	1.12	1.18	1.30	1.42	-
1	1.06	1.26	1.25	1.26	1.10
2	1.14	1.32	1.34	1.36	1.11
3	1.09	1.20	1.30	1.54	1.15
4	1.04	1.18	1.18	1.71	1.08
5	-	-	-	1.86	1.03
6	-	-	-	2.06	0.98
7	-	-	-	1.98	1.02

Table D3. Total Plasma Bilirubin (mg/dl).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	0.05	0.05
-2	0.15	0.2	0.2	0.1	-
-1	0.15	0.1	0.2	0.1	0.1
0	0.2	-	-	0.05	0.05
1	0.1	0.15	0.1	0.1	0.1
2	0.1	0.15	0.15	0.1	0.1
3	0.15	0.1	0.15	0.1	0.1
4	0.15	0.2	0.15	0.1	0.1
5	-	-	-	0.05	0.1
6	-	-	-	0.05	0.1

Table D4. GOT (S/F U/ml).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	88	104
-2	83	77	90	100	98
-1	57	76	119	-	-
0	53	89	153	108	80
1	52	84	69	100	81
2	44	85	79	90	75
3	42	71	60	84	82
4	38	54	60	88	81
5	-	-	-	70	74
6	-	-	-	86	73

Table D5. BUN (mg/dl).

Day	Sheep ID				
	BF	11	13	10	16
-2	-	9	15	21.7	24.1
-1	16	8	11	20.9	27.3
0	22	13	11	23.9	-
1	-	-	-	25.1	25.7
2	-	-	-	22.9	26.5
3	17	15	12	19.2	29.0
4	19	24	14	22.0	31.5
5	-	-	-	20.2	28.1
6	-	-	-	26.6	28.3
7	-	-	-	14.5	24.8

Table D6. Creatinine (mg/dl).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	-	-
-2	.92	.78	.95	.84	.94
-1	.83	.75	.97	.72	.90
0	.95	.76	.85	.77	-
1	.85	.92	.83	.77	.86
2	.92	.82	.95	.83	.88
3	.82	.80	.96	.86	.94
4	.90	.95	.98	.78	.87
5	-	-	-	-	.99
6	-	-	-	.84	.97
7	-	-	-	.75	.86

Table D7. WBC (x 10^3/ul).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	6.8	5.7
-2	8.5	7.2	7.9	8.8	6.8
-1	10.5	6.3	7.8	7.0	5.8
0	9.8	6.3	5.4	6.3	4.8
1	-	-	-	13.9	4.7
2	-	-	-	11.0	4.9
3	7.4	7.3	6.7	12.6	4.6
4	8.8	7.4	5.8	-	-
6	-	-	-	7.3	5.9

Table D8. Hematocrit (%).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	36.5	35.5
-2	34	37	38	35	26.5
-1	32	35	38	36.5	29.5
0	28	38	35	33	29
1	29.5	35.5	35	35	30
2	33	39	35	34	26.5
3	33	38	27	33.5	25.5
4	31	43	33	33	28
5	-	-	-	28	31
6	-	-	-	34	34

Table D9. Hemoglobin (g/dl).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	12.6	12.5
-2	10.8	12.5	12.3	10.0	8.0
-1	10.6	11.4	12.1	11.0	9.0
0	10.0	13.5	11.6	11.3	9.0
1	9.0	10.0	10.8	10.5	9.0
2	9.5	11.5	10.0	9.8	7.3
3	11.1	12.5	8.8	9.5	7.5
4	10.7	15.2	11.0	10.0	8.8
5	-	-	-	9.5	8.8

Table D10. RBC (x 10^6/ml).

Day	Sheep ID				
	BF	11	13	10	16
-3	-	-	-	10.54	9.54
-2	9.44	10.82	10.96	10.70	7.90
-1	9.26	9.90	10.33	9.90	8.32
0	8.84	11.46	9.86	9.63	8.38
1	-	-	-	9.69	8.30
2	-	-	-	9.22	7.75
3	9.39	10.66	9.39	8.37	6.79
4	9.96	12.52	9.30	-	-
6	-	-	-	8.65	8.56

Table D11. Fecal Output (g/24 hr).

Day	Sheep ID				
	BF	11	13	10	16
-3 to 0	1425	694	228	1141	1214
1	1401	1180	402	1259	1601
2	1392	1060	470	1248	1672
3	1283	983	463	781	2164
4	1124	165	909	-	2020
5	-	-	-	388	1457
6	-	-	-	532	1329

Table D12. Fecal Copper Excretion (mg/24 hr).

Day	Sheep ID				
	BF	11	13	10	16
0	7.44	4.41	1.25	11.59	11.10
1	5.61	7.33	1.26	10.80	12.49
2	7.98	6.29	2.00	11.58	15.51
3	8.23	6.12	2.17	7.77	17.44
4	8.51	1.63	3.33	-	15.15
5	-	-	-	4.60	10.75
6	-	-	-	5.57	10.09

Table D13. Urine Output (ml/24 hr).

Day	Sheep ID				
	BF	11	13	10	16
-3 to 0	453.3	1383.3	373.3	988.3	988.6
1	967	1030	650	950	1190
2	950	770	490	1735	1240
3	920	780	450	1400	1200
4	1030	380	480	850	1400
5	-	-	-	800	1070
6	-	-	-	600	1870
7	-	-	-	780	2080

Table D14. Urinary Copper Excretion (mg/24 hr).

Day	Sheep ID				
	BF	11	13	10	16
0	0.5	1.41	0.33	0.88	0.38
1	1.64	1.48	3.18	1.40	1.31
2	2.97	2.19	2.32	1.96	1.55
3	0.36	1.82	1.72	1.63	-
4	1.52	1.06	1.11	1.00	1.06
5	-	-	-	0.42	0.58
6	-	-	-	0.50	1.26
7	-	-	-	0.37	0.99