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SOME ASPECTS OF COPPER TOXICITY IN SHEEP
GRAZING NEW ZEALAND PASTURES

A thesis presented in partial fulfilment of
the requirements for the degree of
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at Massey University.

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ABSTRACT

Several authors have suggested a role for different predisposing factors in cases of copper toxicity, but any association between these factors and biochemical changes in the sheep have not been studied. Therefore the objective of this study was to look for the changes that occurred in sheep treated regularly with a parenteral form of copper. After identifying some of these parameters, further sheep were subjected to stressors that may be encountered in normal farming practice and the changes in response to copper treatment were compared to those of sheep not subjected to that stressor but treated with the same dose of copper.

Eleven Romney rams were regularly monitored to measure any changes that occurred in certain biochemical parameters when the sheep received a weekly subcutaneous injection of 50mg of copper calcium edetate. The biochemical parameters included serum levels of sorbitol dehydrogenase (SDH) and serum glutamate oxaloacetate transaminase (SGOT), blood copper concentration, and wool copper content. At fortnightly intervals, liver biopsy samples were collected for an estimation of the copper content and for a histopathological examination.

It was found that the activity of SDH and SGOT in the serum became elevated as the liver accumulation of copper increased. SDH levels were the first to change and became elevated up to six weeks earlier than SGOT. Although the pathological changes in the hepatocytes developed progressively, their severity and extent did not have a direct relationship to liver copper content. There was no significant change in the copper content of either blood or of wool during the period that the liver copper content increased.

The determination of indicators of early changes in cases of toxicity allowed stressors to be superimposed on copper therapy to assess whether such stressors might influence copper toxicity. The sheep involved received 3mg Cu/kg bodyweight as copper calcium edetate on the premise that sheep of low bodyweight might receive a double injection of the currently recommended dose of 50 mg per sheep. The same biochemical parameters of SDH and SGOT activity, blood copper concentration and liver copper content were used to assess the potentiating effects on copper toxicity of sheep first treated with copper calcium edetate parenterally and then subjected to various stressors. For this purpose 56 sheep, with additional control animals where appropriate, were divided into their respective groups. The stressors included dehydration by removing 25% of blood volume, starvation by fasting for 48 hours, exposure to cold (5°C) for 5 days, and exposure to heat (40°C) for 5 days. Other sheep were either immersed in an organophosphate insecticide, or treated with thiabendazole anthelmintic at a dose rate of 100 mg/kg bodyweight. Pregnant sheep, and others heavily parasitised (e.p.g. > 1570) were similarly treated.

The stressors of dehydration and cold, pregnancy, the application of insecticide or the administration of anthelmintic showed no evidence of enhancing the toxic effects of copper. However the stressors of starvation, heat, and parasitism did potentiate toxicity and resulted in approximately half of the sheep in each group dying from copper toxicity.

A further series of experiments used 60 sheep, divided into 14 groups; each group being given a different schedule of copper administration which consisted of one of the stressors and/or one of a series of formulations of copper consisting of salts made up in various bases. Blood samples were collected hourly for 16 hours and the rate of change of blood copper concentration was measured. In the sheep that were starved, the rate of change of blood copper concentration increased to 0.141 mg Cu/l/hr for animals starved for 72 hours in comparison with a rate of 0.056 mg Cu/l/hr for animals

given access to food and water. Those sheep that received 50 mg of copper calcium edetate in either of two proprietary formulations; one containing polyvinyl pyrrolidone (PVP) and the other without PVP but the same dose contained in half the volume, showed a mean blood copper concentration rate of increase of 0.017mg Cu/l/hr. An increase in the dose to 100mg Cu increased the rate of uptake of copper to 0.022mg Cu/l/hr, whereas a 50mg dose diluted in an equivalent amount of water showed an increase in the rate of translocation of copper to 0.036mg Cu/l/hr. The four sheep subjected to heat stress or given copper by mouth as copper edetate at a dose rate of 0.33mg/kg showed a blood copper concentration increase to 0.025mg Cu/l/hr, whereas 7gm of oral copper oxide needles administered to three 50kg sheep did not produce any increase in blood copper concentration during the period of study.

Starved sheep also showed changes in their blood concentrations of glucose and albumin. Blood glucose reduced from a mean of 4.7gm/100ml to a mean of 2.4gm/100ml in the nine sheep starved over 72 hours, plasma albumin increased from 1.30gm/100ml to 2.26gm/100ml, and total protein rose by 10.2%.

Deaths following the administration of copper therapeutically have been reported on many occasions. Therefore it was decided to measure the effects of copper therapy on the liver copper storage of sheep which initially had a range of liver copper concentrations. A "copper deficient" farm which regularly reports lambs with enzootic ataxia, and a "copper sufficient" farm with no reported signs of copper deficiency in sheep, were selected. Two hundred sheep on the copper deficient farm and fifty sheep on the copper sufficient farm were treated once annually with 50mg of copper calcium edetate given subcutaneously. This dose was adequate to maintain the liver copper content of all treated sheep on the copper deficient farm above 70 ppm Cu D.M. However in the sheep grazing the copper sufficient farm, liver biopsy samples indicated that copper, apparently surplus to requirements, was stored in the liver resulting in copper concentrations in all sheep in excess of 510 ppm Cu D.M.

Another study measured the uptake of copper by the liver in groups of four sheep of four different breeds common in New Zealand. These breeds were the Border Leicester, N.Z. Romney, Suffolk and Merino. There was no significant difference between the former three breeds, but the Merinos retained less copper in their livers after grazing pasture for 3 months (88ppm vs 164ppm), and also following administration of copper by subcutaneous injection (215ppm vs 330ppm).

The results of this work indicate that certain common stressors met with in everyday sheep management, may enhance copper toxicity. Copper should never be administered to sheep unless the requirement has been confirmed, and at the time of administration particular attention should be paid to avoiding those circumstances that might lead to starvation of the sheep.

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CHAPTER 1: LITERATURE REVIEW

Historical

Chronic copper poisoning in farm animals was first recorded in 1883 by Ellenberger & Hofmeister who induced the condition experimentally. The first confirmed field case in sheep occurred in animals grazing in orchards that had recently been sprayed with Bordeaux mixture (Schaper & Luetje, 1931; Beijers, 1932). Since that time clinical and experimental cases have been reported from natural and accidental causes and from most countries of the world.

The first report of possible copper toxicity in New Zealand sheep was made by Hopkirk, (1931), who described cases of 'epizootic jaundice' which occurred in January and March. These sheep were grazing land that was growing excessive clover. In the subsequent reports (Hopkirk, 1932, 1933, 1934 & 1937) deaths due to epizootic icterus were reported and the sheep referred to in the 1934 outbreak were suspected of having eaten ragwort (Senecio jacobea). Fitch, (1938), analysed the livers of sheep which had died of enzootic icterus, and he found that these had copper concentrations as high as 3,280 ppm D.M.

Subsequent work at Wallaceville by Buddle in 1939 showed that sheep dosed with 100ml of 1% CuSO_4 daily, developed signs and showed post-mortem lesions similar to those described in epizootic jaundice. The liver analysis from these sheep revealed 2,940, 4,030, 3,500, & 1,950 ppm D.M. of copper, (Hopkirk, 1939).

Copper as an essential element

Soil as a source of copper

The earth's crust contains between 45 ppm Cu and 70 ppm Cu (Hodgson, 1963; Norrish, 1975). Most soils of the world contain about 20 ppm but estimates ranging from 2 - 1,000 ppm have been recorded (Allaway, 1968; Aubert & Pinta, 1977). In New Zealand, soils commonly contain about 17.5 ppm of copper. Some of these soils have levels as low as 2 ppm but the upper limit rarely exceeds 25 ppm (Wells, 1957), apart from the yellow brown loams and brown granular loams and clays derived from andesitic ash which have a higher copper content (Rolt, 1962).

The copper content of the soil depends on the parent material from which it was derived (Ermolenko, 1972), and on the amount of weathering, that has subsequently taken place. The greater the amount of weathering; the lower the copper content (Wells, 1957; Ermolenko, 1975).

Copper may be bound in the soil (a) in a water-soluble form, (b) as exchangeable ions, (c) in a non-exchangeable form as an organic complex, (d) in association with iron, aluminium and sometimes manganese oxides, or (e) as a constituent of crystal lattices of other sand-, silt-, and clay-sized minerals (Le Riche *et al.*, 1963; Mitchell, 1972; Ermolenko, 1975; Norrish, 1975; Aubert & Pinta, 1977; McBride, 1981). Up to 99% of copper in soil solution may be complexed with organic matter (Hodgson *et al.*, 1966; Mitchell, 1972). This formation of insoluble organic complexes may act as a reserve supply of copper for the plant (Ermolenko, 1972), and such copper may be liberated in a form accessible to plants in conditions liable to cause the copper to precipitate (e.g. in calcareous soils). The complexes may act as a transport and carrier agent of copper for plant use, or they may reduce the concentration of available ionic Cu^{++} to a non-toxic level when excess copper is

present (McBride, 1981; Stevenson & Fitch, 1981).

The mobility and availability of copper is related to soil texture, drainage, redox potential, pH, and organic matter status (Thornton, 1979).

Wet soils are usually anaerobic and they tend to convert copper to various insoluble sulphides. If the soil is low in organic matter, the soluble copper compounds are leached out (Ermolenko, 1972). Wet-dry cycles tend to increase the amount of exchangeable copper in chelated form (Ng Siew Kee & Bloomfield, 1962) but this does not necessarily impair its availability to plants (Tiller et al., 1972; McLaren et al., 1974). Drainage of wet soils may limit the availability of copper by favouring the formation of more highly oxidized forms (Allaway, 1968), but drainage may still increase the uptake of copper by clovers by as much as twenty-five percent (Mitchell, 1972).

Increasing soil acidity by the application of nitrogen fertilizers (Ermolenko, 1975; Loneragan, 1975), increases the mobility of copper (Ermolenko, 1972; Loneragan, 1975), whereas increasing the soil pH with lime decreases the mobility of copper (Ermolenko, 1972) 1975) by increasing the adsorption of Cu^{++} ions on to iron and manganese oxides and clay lattices (Murray et al., 1968; Murray, 1975; James & Barrow, 1981). It has been reported that overliming can render copper unavailable in those soils liable to induce copper toxicity (Peech, 1941; Aubert & Pinta, 1977).

The application of copper fertilizers to soils may increase the concentration of copper in plants (Wells, 1957; Branion, 1960; Peive, 1959). The increase has been found to be as high as 30% in some instances (Mitchell et al., 1957). As copper is not a mobile element in soil (Hodgson, 1963) the effect of copper supplementation depends on the establishment of a large number of copper depots in the soil (Gilkes, 1981), and on the soil pH, fertilizer particle size and the root interception by the plant.

In the diagnosis of copper deficiency in either plants or animals, the measurement of the total amount of copper in the soil is of little value, as it is seldom correlated with the amount of copper available to plants (Le Riche et al., 1963; Mitchell, 1974). It is the extent to which it has been mobilized and is present in the soil in an available form that is significant (Mitchell, 1972).

Plants as a source of copper

Copper is required by the more highly developed plants for incorporation into enzymes that assist in respiration, photosynthesis and the formation of proteins required for lignification. It is also involved in anabolic metabolism, cellular defense mechanisms and hormone metabolism (Nicholas, 1975; Walker & Webb, 1981).

The amount of copper absorbed by the roots of the plant depends on the mass flow of copper in soil solution, the diffusion of copper down the osmotic gradient, and the amount of root interception (Mitchell, 1972; Ermolenko, 1975; Loneragan, 1975; Graham, 1981; Jarvis, 1981). The root interception is determined by the root length per plant, and by the density of root hairs and the amount of root exudate secreted to mobilise Cu^{++} from the soil organic complexes (Ermolenko, 1972; Loneragan, 1975; Norrish, 1975; Graham, 1981). The roots of plants accumulate copper in a bound form in the cell walls (Graham, 1981; Jarvis & Jones, 1979), and when soil copper content is high, they may contain 10-100 times as much copper as in the plant solution (Chandry & Loneragan, 1970). This excess copper is not translocated to the plant shoots, unless the roots are damaged, in which case copper may be released to enter the plant (Graham, 1981).

Plant tissues normally contain 5-20 ppm D.M. of copper. Copper deficiency usually occurs when the concentration falls below 4 ppm while toxicity occurs when levels rise above 20 ppm (Jones, 1972; Robson & Reuter, 1981). In New Zealand very few areas grow pastures which contain more than 13 ppm of copper (Cunningham et al., 1956;

Rolt, 1962). The young shoots of plants contain the highest amounts of copper but levels decrease steadily as the plant matures (Adams & Elphick, 1956; Cunningham et al., 1956; Allaway, 1968; Mitchell, 1972; Loneragan, 1975; Loneragan, 1981). This movement of copper from old leaves during senescence parallels the loss of nitrogen until at full senescence, leaves may only contain 20% of the original copper content (Loneragan, 1975; Loneragan et al., 1980; Loneragan, 1981). As the seed heads of plants mature they gain copper at a rate similar to the decline in the concentration of copper in the leaves and stems (Loneragan, 1981).

Legumes have a higher affinity for copper than do grasses (Beeson & MacDonald, 1951; Adams & Elphick, 1956; Mitchell et al., 1957; Branion, 1960; Gladstones & Loneragan, 1970; Patil & Jones, 1970), especially when they are growing in soils of higher copper content or in soils that have been supplemented with copper.

The application of copper fertilisers to copper deficient soils will increase the copper content of plants growing in that soil (Loneragan, 1975), but copper application will have little effect on plants growing in soils containing adequate copper levels (Mitchell et al., 1957; Allaway, 1968). Fertilisers such as nitrogen that promote plant growth and alter pasture composition tend to reduce the pasture copper content (Loneragan, 1975; Reith et al., 1979).

High concentrations of copper are toxic to most plants. The symptoms of copper poisoning include reduced shoot vigour, poorly developed and discoloured root systems, and leaf chlorosis (Smith & Specht, 1953; Delas, 1963; Reuther & Labanauskas, 1966; Daniels et al., 1972). Toxicity commonly occurs in very acid soils ($\text{pH} < 5$) which have low cation exchange capacity (Reuther & Smith, 1954; Leeper, 1978), or it may be induced by the application of copper to soils with a low organic matter content (Delas, 1963; Page, 1974; Purves, 1977).

Many plant species, especially herbaceous plants, can tolerate high copper levels by immobilising copper complexes in cell vacuoles or in non-diffusible metal-protein complexes, or by excluding the uptake of the metal (Woolhouse & Walker, 1981).

The copper content of plants should be measured by the analysis of plant material, and whenever concentrations in whole shoots exceed 20 ppm D.M., the plant must be treated as a potential source of copper toxicity to animals (Robson & Reuter, 1981).

Absorption of copper by the animal

There have been many studies on the absorption of copper by sheep and cattle. Most of these have dealt with circumstances of the normal physiological process and those involving toxicity. There are clearly species and age differences of importance. The newborn lamb absorbs copper very efficiently. Immediately after birth almost 100% of the lamb's copper intake is absorbed, but uptake decreases to 10% by weaning (Suttle, 1973; Suttle, 1975; Suttle, 1979). This decrease in absorption is due to both the lowering of the concentration of copper in the ewe's milk as lactation advances (from 1.2-1.4 ppm in colostrum to 0.05-0.29 ppm at weaning) (Beck, 1941; Polidori, 1960; Ashton & Williams, 1977; Lonnerdal et al., 1981), and the decreased absorption of copper via the ileal mucosa (Suttle, 1975).

The lamb foetus begins to accumulate copper in its liver at the twelfth week of gestation, while maternal liver stores start to decrease in the fourteenth week of pregnancy (Russanov et al., 1981). The newborn lamb has low concentrations of liver copper but rapidly accumulates copper in the abundant mitochondrial hepatocuprein (Suttle, 1975).

Adult sheep absorb less than 10% of dietary copper (Suttle, 1979), and only about 5% of the dietary intake is stored in the liver (Dick, 1954; Hemingway & MacPherson, 1967; Suttle, 1973; Lee,

1974; Moodie, 1975). The absorption of copper appears to be a physiologically controlled process (Neethling et al., 1968), as during lactation ewes temporarily increase their efficiency of absorption (Suttle, 1979). A similar phenomenon is found in sheep with hypocupraemia (Neethling et al., 1968; Hill et al., 1969), and it was proposed by Evans & Johnson (1978) and by Bremner (1981) that it is the protein metallothionein in the intestinal mucosa that is largely responsible for the control of copper. Metallothionein determines the rate at which copper is transported across the intestinal mucosa.

Copper can only be absorbed in the ionic form by attachment to a binding site on metallothionein (Mills, 1961).

Metallothionein provides binding sites for copper within the intestinal mucosa and acts as a temporary storage site pending subsequent absorption or excretion of this element (Evans, 1973), but it does have an upper threshold level (Saylor et al., 1980). At the serosal surface of the intestine the copper dissociates from metallothionein and either diffuses directly into the plasma as ionic copper or becomes bound to either albumin or a copper-amino acid complex for transport to the liver via the portal circulation (Figure 1.1) (Bush et al., 1956; Beaton & McHenry, 1964, Evans, 1973).

The efficiency with which copper is assimilated from the diet depends on the solubility of the copper (Mills, 1961), which in turn is largely dependent upon pH (Bremner, 1970; Kirchgessner & Grassman, 1970). In the rumen most of the copper is present either in an insoluble form or is bound to receptors in the rumen microorganisms (Mills, 1961; Bremner, 1970). As the copper complexes are more stable at lower pH, abomasal absorption is minimal, but with the rising pH of ingesta distally, copper is freed and absorbed in the ileum (Bremner, 1970) and in the large intestine (Grace, 1975). The amount absorbed in the ileum is small, and is similar in amount to that secreted in the biliary and pancreatic secretions (Grace, 1975).

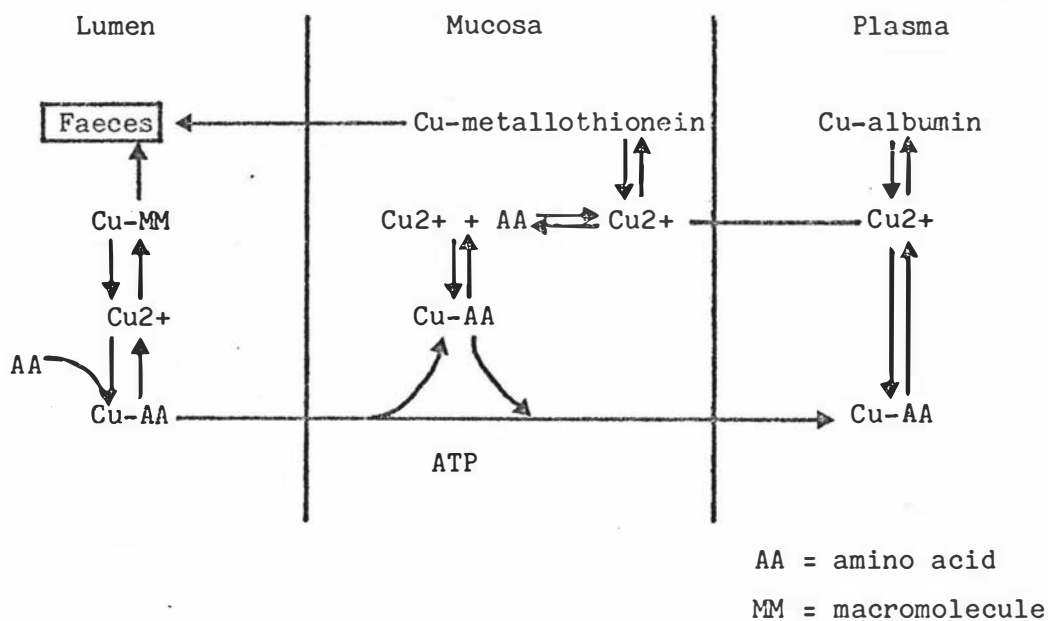


Figure 1.1: Intestinal absorption of copper (Evans, 1973)

Approximately 72% of ingested copper is excreted in the faeces and about 2% in the urine (Scheinberg & Sternlieb, 1960; Neethling *et al.*, 1968; Evans, 1973; Grace, 1975; Grace & Gooden, 1980). The remainder of endogenous copper is lost in the secretions of saliva (Bertoni *et al.*, 1976; Stevenson & Unsworth, 1978; Grace *et al.*, 1981), bile (van Ravesteyn, 1944; Grace & Gooden, 1980), and pancreatic juices (Grace & Gooden, 1980); none of which is reabsorbed from the gastrointestinal tract as the copper ions are bound in organic complexes (Grace, pers. comm.).

The difference in the absorption rate and in the excretion rate between species may affect the copper requirements and the copper reserves of these animals. Sheep are more susceptible than other domestic ruminants to copper accumulation and possible toxicity, for three reasons. First, they have less control of the intestinal copper homeostatic mechanism (Bremner & Davies, 1979), secondly, they have limited capacity to excrete copper from the liver (Beck, 1963; Neethling 1968; Corbett *et al.*, 1978; Bremner, 1981), and thirdly, their lysosomes are unable to sequester large amounts of copper

(Corbett et al., 1978).

Metabolism of copper by ruminants

Copper is essential for the metabolism of most plant and animal cells (Scheinberg & Sternlieb, 1960). In the animal body it is an essential part of many enzymes and structural and carrier proteins. The highest concentrations of copper are found in the liver, brain, heart and kidney. The lung, intestine and spleen carry intermediate levels, while the endocrine glands, muscle and bone have the lowest levels (Adelstein & Vallee, 1962; Evans, 1973).

After intestinal absorption, much of the copper is rapidly deposited in the liver. Deposition occurs within minutes of absorption and continues for several hours (Scheinberg, 1961). In the liver, copper undergoes a chain of metabolic processes preparing copper ions for subsequent incorporation into proteins for storage, transport and excretion of copper. There are three distinct pathways involved in this process: (i) the preparation of copper for secretion into bile for excretion, (ii) the temporary storage of copper, and (iii) the incorporation of copper into caeruloplasmin for distribution throughout the body (Evans, 1973).

Biliary copper enters the bile canaliculi bound to amino-acid (Evans, 1973), and its resorption is negligible.

Copper is stored in the liver hepatocytes: the distribution being 20% in the nuclear fraction, 10% in microsomes, and 20% in the large granules of mitochondria and lysosomes. The remainder is stored in the cytosol as either copper-dependent enzymes or metallothionein (Evans, 1973; Bloomer & Sourkes, 1974; Davies & Wahle, 1978; Saylor & Leach, 1980; Saylor et al., 1980; Russanov et al., 1981). The copper concentration of the subcellular fractions of the liver is related to the total copper content of the liver rather than to the physiological state of the animal such as age, species or copper status (Gregoriadis & Sourkes, 1967). Once

concentrations reach a certain threshold, any copper surplus to requirements is sequestered into the lysosomes so that copper ions are not available to initiate toxic effects (Lindquist, 1968; Worwood & Taylor, 1969; Evans *et al.*, 1973; Sternlieb & Goldfischer, 1976) (Figure 1.2). As the liver becomes saturated with copper then the kidneys become the secondary site of copper deposition (Walshe, 1968).

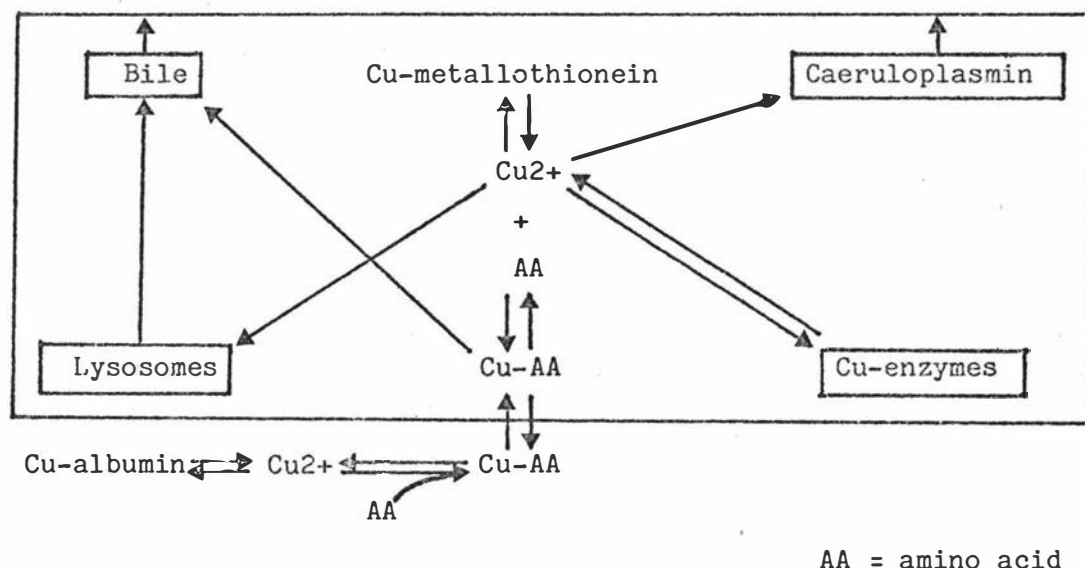


Figure 1.2: Copper homeostasis in the normal hepatocyte
(Evans, 1973)

Caeruloplasmin, a globulin, is a multifunctional metalloprotein responsible for copper transport. It also facilitates the mobilization of iron from iron storage sites to plasma for haem synthesis, and acts as a regulator of circulating biogenic amine levels through its oxidase activity (Beaton & McHenry, 1964; Evans, 1973; Evans & Abraham, 1973; Frieden & Hsieh, 1976; Mills *et al.*, 1976; Frieden, 1978). Caeruloplasmin is synthesized in the liver, and its rate of formation depends on the hepatic copper concentration (Bush *et al.*, 1956; Evans, 1973; Moodie, 1975). Thus, it aids in the regulation of hepatic copper concentration. It has a half-life of about 50 hours in plasma (Frieden & Hsieh, 1976).

Blood contains copper in five separate fractions: in erythrocyte superoxide dismutase which contains 60% of erythrocyte copper, in an erythrocyte copper complex, in plasma caeruloplasmin which comprises 60-95% of plasma copper (Shields et al., 1961; Westerfield, 1961; McCosker, 1968a; Weser, 1974), in albumin-copper, and bound to plasma amino acids (Brown et al., 1968; Evans, 1973; Moodie, 1975; Frieden & Hsieh, 1976). The erythrocyte and plasma copper contents are approximately equal (Westerfield, 1961). The total content of copper in the erythrocyte does not fluctuate in spite of variations in the copper status of the animal (Evans, 1973) and erythrocytes are not involved in copper transport (Evans, 1973; Frieden & Hsieh, 1976).

Copper is incorporated into the molecular structures of five major enzymes, and several less important ones, as well as being part of several proteins and amino-acids (Evans, 1973). Caeruloplasmin is a major copper enzyme because of its ferroxidase activity and involvement in iron transport (Table 1.1).

Cytochrome oxidase is the terminal enzyme in the oxidative phosphorylation process and is sited in mitochondria (Scheinberg & Sternlieb, 1960; Gallagher & Reeve, 1971; Evans, 1973; Mills et al., 1975). Dietary copper deficiency may result in a 40-50% reduction in the rate of cytochrome oxidation activity (Davies & Wahle, 1978) affecting especially the liver and intestinal mucosa (Boyne, 1978), and myelin formation in the central nervous system (Gallagher & Reeve, 1971; Evans, 1973). Monamine oxidase (lysyl oxidase) is the enzyme required for the cross-linkage of the peptide chains of collagen and elastin (Bornstein et al., 1966; Partridge, 1966; Carnes, 1971; Mills et al., 1976; Harris & Rayton, 1978) which maintain the structural integrity of both vascular and skeletal tissue (Evans, 1973). Copper deficiency is characterized by fragility of the skeletal system (Carnes, 1971; Evans, 1973).

Enzyme	Activity	Source	Function
Caeruloplasmin	ferroxidase	plasma	iron transport
Cytochrome oxidase	terminal oxidase	mitochondria	energy metabolism & phosphorylation
Lysyl oxidase	peptide cross-linkage	aorta & cartilage	collagen & elastin formation
Tyrosinase	oxidase	melanocytes	tyrosine to melanin
Superoxide dismutase	dismutase	all aerobic cells	$O_2 + O_2 + H_2O \rightarrow H_2O_2 + O_2$
Dopamine-B-hydroxylase	oxygenase	adrenal gland	dopamine to nor-epinephrine

Table 1.1: Essential copper-containing enzymes and their functions.

Tyrosinase is an oxidase required for the conversion of tyrosine to melanin needed for pigmentation (Scheinberg & Sternlieb, 1960; Walshe, 1968; Evans, 1973).

Superoxidase dismutase (which includes all the cupreins) is present in all aerobic cells (Weser, 1974; Fridovich, 1975). The enzyme catalyses the dismutation of superoxide free radical anions to hydrogen peroxide and oxygen (McCord & Fridovich, 1969b; Evans, 1973; Fridovich, 1975b). It is the primary defence mechanism against the toxic singlet oxygen radical (Evans, 1973; Weser, 1974; Fridovich, 1975a).

Copper requirements by the sheep

If copper associated with the liver is excluded, a fully fleeced sheep, contains about 60mg of copper; each kilogram of bodyweight containing 0.8mg of copper and each kilogram of wool containing 6-8mg of copper (Grace, 1983), to 9-11mg of copper (Cunningham & Hogan,

1958). A newborn lamb contains a total of 10mg of copper (Pryor, 1964; Williams et al., 1978). The nett requirements for maintenance rarely exceed 4ug of copper per kilogram of bodyweight per day and show no relationship to metabolic rate (Suttle, 1974b; Smith, 1981; Grace, 1983). Growth requires approximately 1.1mg copper per kilogram of bodyweight increase (Smith, 1981; Grace, 1983). The foetus requires 2.8mg of copper per kilogram of bodyweight (Grace, 1983), and a lactating ewe requires an extra 0.3mg copper for every litre of milk produced (Grace, 1983).

Factors influencing copper absorption and metabolism

Newborn lambs have a higher total copper concentration than their dams; up to 50% of their copper being present in the liver (Cunningham, 1931; Adelstein & Vallee, 1962; Pryor, 1964; Hartmann et al., 1978). Such copper is stored as neonatal hepatic mitochondriocuprein which has either a storage or a detoxifying role, or both (Walshe, 1968; Porter, 1974). Plasma copper levels in the neonate are lower than those of the dam, as the foetus is unable to synthesize caeruloplasmin (McCosker, 1968; Walshe, 1968; Moodie, 1975) and caeruloplasmin does not cross the placental barrier (Sternlieb & Scheinberg, 1960). However caeruloplasmin levels of the lamb rise rapidly to adult levels within seven days of birth (Howell et al., 1968; McCosker, 1968), while foetal mitochondriocuprein liver reserves decrease (Wiener et al., 1974). Young lambs absorb copper very efficiently while they are on a liquid diet. This high rate of absorption is due to the higher absorptive capacity of the large intestine, and the lack of interference by sulphide from rumen microflora. Thus the increased requirements of a rapidly growing lamb on a low copper-content milk diet are satisfied (Suttle, 1982b).

The amount of copper absorbed from natural food varies considerably, depending on the type of food and the season of the year. The copper contained within stored fodder such as hay or silage is absorbed at a higher rate than that of pasture (Table 1.2) (Mills, 1961; Suttle, 1979; Suttle, 1981a; Suttle, 1982b).

Feed	% Absorption
summer pasture	2.3
autumn pasture	1.2
hay	7.2
silage	4.9
leafy brassicas	12.8
root brassicas	6.7

Table 1.2: Copper absorption from various feeds (Suttle, 1981a)

Although MacPherson and Hemingway (1965) considered that protein retards the absorption of copper, it is more probable that the high sulphur content of most protein diets is the factor limiting copper absorption.

Metabolic processes can adapt to the variations of copper availability as can the excretory and absorptive mechanisms (Kirchgessner *et al.*, 1981). During periods of high copper requirements there can be, for example, active bone resorption and bone demineralization to meet the copper demands of the animal (Ljubashevsky, 1978; Anon, 1981). Higher plasma levels of copper are seen in response to increased oestrogen levels, as in ovulation (Hidiriglou *et al.*, 1982), pregnancy (Scheinberg & Sternlieb, 1960; Scheinberg, 1961; Adelstein & Vallee, 1962; Pryor, 1964; Moss *et al.*, 1974; Howell, 1968; Russanov *et al.*, 1981). Plasma concentrations of copper may also increase following the use and release of corticosteroids (Henkin, 1974; Andrewartha & Caple, 1978)

There is considerable genetic variation between breeds and strains of sheep in their ability to absorb copper. This has been shown from the variable incidence of enzootic ataxia recorded in different breeds of sheep (Wiener, 1966; Wiener & Field, 1969;

Poole, 1970; Wiener, 1971), and in their susceptibility to copper toxicity (Edgar et al., 1941; Marston & Lee, 1948b; Luke & Weirman, 1970; Wiener & Field, 1971; Luke & Marquering, 1972; Wiener, 1973; Schmitten et al. 1978; van der Berg et al., 1983). The maternal effect may influence copper levels for the first twelve weeks of life (Wiener, Herbert & Field, 1976; Wiener et al., 1977; Wiener et al., 1978), whereas after weaning the breed of the sire strongly influences the concentration of liver copper in the progeny (Wiener, Hayter & Field, 1976; Suttle, 1982b; Woolliams et al., 1982). There is a greater seasonal variation in copper uptake and storage in those strains or breeds of sheep that have a lower ability to accumulate copper (Wiener et al., 1969; Wiener et al., 1970; Hayter et al., 1973). This difference between breeds diminishes as the molybdenum content of the diet is increased (Suttle, 1981b). The genetic variation in copper levels between breeds of sheep is due to differences in the absorption of copper rather than its utilisation or excretion (Herbert et al., 1978; Wiener et al., 1978). The rumen is probably the site at which the change in copper availability is initiated because dosing sheep with copper oxide needles into the abomasum eliminates any differences determined by the breed of sheep (Wiener, 1980).

In ruminants the presence of certain elements interferes with the absorption and utilisation of copper and may result in the development of copper deficiency. When such inhibiting elements are not present or are in low concentrations, copper toxicity may still be induced. These elements either inhibit the absorption of copper or compete directly with copper at its copper binding sites within the animal.

In the rumen molybdenum combines with sulphur to form insoluble thiomolybdates which may form copper thiomolybdates and render copper insoluble. It has been shown that levels of molybdenum in the diet in excess of 5 ppm D.M., if present with sulphur ions, may seriously affect the uptake of copper by ruminants (Dick, 1953; Dowdy & Matrone, 1968a & 1968b; Smith et al., 1968; Spais et al., 1968;

Suttle & Field, 1968a, 1968b, 1968c; Suttle, 1974b, 1974c; Smith & Wright, 1975; Suttle, 1975; Suttle & Field, 1983).

Other ions which may affect the absorption of copper at this site include zinc (Evans 1973; Bremner, 1979; Reynolds, 1979), iron (Dick, 1954; Anthony & Nix, 1965; Sourkes et al., 1968; Standish et al., 1971), cadmium (Evans, 1973; Hennig et al., 1974; Doyle & Pfander, 1975; Ghergariu, 1978; Bremner, 1979), lead (Hemingway et al., 1964; Alloway, 1969; Ghergariu, 1978) and calcium (Dick, 1954; Adelstein & Vallee, 1962). Other heavy metals may compete with copper for certain binding sites. These include cadmium (Evans, 1973), silver (Evans, 1973), nickel (Evans, 1973), cobalt (Evans, 1973) and zinc (Evans, 1973; Bremner & Marshall, 1974; Bremner et al., 1976; Bremner et al., 1977). Manganese and molybdenum may also depress copper levels by increasing its urinary excretion (Gubler et al., 1954; Dowdy & Matrone, 1968b).

Ingested soil is a source of many minerals to the grazing sheep (Field & Purves, 1964; Ghergariu, 1978) and during the winter months soil may comprise up to 30% of the diet (Healy, 1969; Suttle et al., 1975). The ingestion of such large quantities of soil may decrease by up to 50% the soluble copper concentration of the duodenal liquor (Healy, 1970).

Copper deficiency of sheep

Many conditions have been diagnosed in ruminants as being the result of copper deficiency, or they have responded to copper therapy. Copper deficiency may affect growth, development of the skeletal and nervous systems, formation of erythrocytes and wool fibres, or the function of leucocytes, the gastrointestinal tract and the reproductive system. These signs may occur singly or in combination and not necessarily in any particular sequence.

Lambs which are raised on milk having a low copper content, have a lower plasma copper concentration and show a reduced growth rate (Lee, 1951a; Whitelaw et al., 1977; Whitelaw & Evans, 1979; Whitelaw et al., 1979; Whitelaw et al., 1981). Weaned lambs provided with a low copper diet supplemented with copper, may achieve good weight gains in comparison to control animals (Bennetts & Beck, 1942; Howell, 1968; Hogan et al., 1971). Under different circumstances copper deficient lambs may continue to have good growth rates but if for any other reason their growth is impaired, the effect is much more dramatic (Lewis, 1970).

Prolonged copper deficiency results in light and brittle long-bones because of the thinning of their cortices (Hogan, 1973; Hidioglou, 1980; Hurley, 1981). Osteoporosis, especially in the central metaphyseal region (Cunningham, 1944; Suttle et al., 1972; Whitelaw et al., 1979; Hidioglou, 1980) and rib fractures (Whitelaw et al., 1979; Hurley, 1981) may also be seen. Sheep with mandibular osteopathy and resulting dental abnormalities, have been found to have low liver copper concentrations (Bruere et al., 1979). The osteoblasts in lambs which have been depleted of copper both in utero and during the suckling period, are particularly sensitive to copper deficiency (Suttle et al., 1972), but they will respond rapidly to an increased copper intake (Doyle, 1979).

Enzootic ataxia is caused by copper deficiency and is the most characteristic manifestation of copper deficiency. It affects lambs up to four months of age and is characterised by hindlimb paralysis, severe incoordination, and sometimes blindness (Bennetts, 1932; Innes & Shearer, 1940; Bennetts & Beck, 1942; Hurley, 1981; Smith et al., 1981). The neuronal changes of enzootic ataxia are present in the brain stem and the grey matter of the spinal cord. The predilection sites are the red and vestibular nuclei, the reticular formation of the brain, and the ventral horns of the spinal cord (Barlow 1960b; Howell et al., 1964; Fell et al., 1965; Howell, 1970; Howell et al., 1981; Smith et al., 1981). Cerebral cavitation occurs in 20-60% of cases with accompanying cerebral

expansion due to the pressure of entrapped cerebrospinal fluid (Roberts et al., 1966; Howell, 1970; Howell et al., 1981). The main central nervous system lesions are the result of hypomyelination associated with a reduction of cytochrome oxidase activity (Barlow et al., 1960b; Barlow, 1963b; Howell et al., 1964; Fell et al., 1965; Howell, 1970; Prohaska, 1981). There are two peak periods for the laying down of myelin. The first is during the last fifty days of pregnancy when the major growth phase of the cerebrum occurs and the second period is during the first five weeks of neonatal life when the spinal cord doubles in size without an increase in cell numbers (Barlow, 1963a; Smith et al., 1977; Howell et al., 1981; Prohaska, 1981). If one twin is affected with enzootic ataxia (also referred to as swayback), then the other twin will develop the condition although not necessarily at the same time (Lewis et al., 1981).

The criteria used for the diagnosis of enzootic ataxia are (i) ataxia in the young or newborn lamb, (ii) cavitation or gelatinous lesions of the cerebral white matter often accompanied by histological evidence of chromatolysis and myelin degeneration in the brain stem and spinal cord, and (iii) low copper status (Barlow et al., 1960a). The copper content of the spinal cord is considered to be the best indicator of the copper status of these animals (Howell, 1970).

Anaemia has been reported in association with copper deficient sheep (Bennetts & Chapman, 1937; Bennetts & Beck, 1942; Suttle & Field, 1967; Hogan, 1973; Whitelaw et al., 1979); as copper is required in ferroxidase to mobilize iron for haemoglobin synthesis (Gallagher et al., 1956 ; Blunt, 1975; Holmes & Dargie, 1975).

The first sign of depletion of copper reserves is often loss of crimp in the fleece (Lee, 1951a; Fearn & Habel, 1961; Suttle & Field, 1967). With further depletion wool production is severely diminished and there is a deterioration in the wool staple with the appearance of straight lustrous fibres typical of "steely wool" (Marston & Lee, 1948a; Lee, 1951a, 1951b; Whitelaw et al., 1979).

The copper deficiency causes an impediment in the keratinization process by reducing cross linkage of disulphide bonds between polypeptide chains (Danks et al., 1972). Achromotrichia also occurs because there is insufficient copper dependent tyrosinase to produce melanin. This may result in either an overall pallor in black or coloured fleeces, or a banding effect on the fibre (Lee, 1951b; Suttle & Field, 1970a; Hogan, 1973).

Copper deficiency may influence the susceptibility of ruminants to infection as it has been shown to impair the phagocytic activity of leucocytes (Boyne & Arthur, 1981; Jones & Suttle, 1981) and the immune response of other mammals (Newberne et al., 1968; Gross & Newberne, 1980; Nauss & Newberne, 1981; Prohaska & Lukasewycz, 1981; Jones & Suttle, 1983).

Bennetts and Beck (1942) observed diarrhoea and loss of condition during late gestation and lactation in ewes of low copper status. This may have been due to partial villus atrophy in the duodenum and jejunum as has been recorded in copper deficient cattle (Fell et al., 1975).

Low plasma copper levels have been reported to reduce libido in rams (Wiener et al., 1976). In ewes on very low copper semi-synthetic diets, barrenness, foetal resorption and abortion have been reported (Howell, 1968; Howell & Hall, 1970; Suttle & Field, 1970a), with the margin in copper status between that causing infertility and that causing production of swayback lambs being a very small one (Suttle & Field, 1970a).

Therapy of copper deficiency

Since the recognition of copper deficiency, many copper compounds have been tested at a variety of dose rates and administered by different routes. The results have varied. In the treatment of whole flocks of animals, the margin between adequate protection and toxicity appears to be narrow.

Harvey and Sutherland (1953) describe the qualities desirable for an injectable copper preparation as (i) producing minimal damage at the site of injection, (ii) causing satisfactory storage in the liver of about 90% of the administered copper (iii) having a safety margin between the therapeutic and toxic dose rate, and (iv) being easily prepared and economical. Therapeutic and safety differences amongst the available compounds depend on the rate of translocation from the injection site to the liver (Mahmoud & Ford, 1981; Suttle, 1981b). Injected copper exhibits a much longer period of therapeutic effect than orally administered copper (Comar, 1950). Only 1.6-1.8% of orally administered copper sulphate is stored in the liver (MacPherson & Hemingway, 1968; ARC, 1980), and 8.3% for copper oxide needles; this is reduced to 3.8% if sheep are being fed on a high molybdenum diet (Suttle, 1981a). On the other hand, the subcutaneous injection of copper edetate as an example, resulted in an 80-90% storage of copper in the liver (Camargo et al., 1962).

The protection from hypocupraemia afforded by a parenteral dose of copper reflects the completeness of transfer from the injection site to the liver. The speed of translocation may determine the extent of any local tissue reaction. However it may be difficult to combine the properties of rapid translocation with an adequately slow arrival at the storage site (Suttle, 1981b).

Deaths may occur in association with the use of parenteral compounds, due to rapid translocation, but deaths only occur in certain flocks (Ishmael et al., 1969; Hendy & Evans, 1977; Gardiner, 1978). To reduce the risk of losses, it is recommended that "stress" at the time of injection should be reduced and that other animal remedies that are detoxified by the liver are not used at the same time as the copper injection (Hendy & Evans, 1977).

Lambs are more susceptible than adult sheep to copper toxicity yet a non-toxic dose of parenteral copper may be insufficient to prevent delayed swayback (Lewis et al., 1981). Copper oxide needles given to lambs will elevate plasma copper concentrations (Whitelaw et

al., 1980), but will not alleviate the clinical condition affecting lambs (Whitelaw et al., 1982). To prevent swayback in lambs a relatively high dose of some copper compound must be given to the ewe during mid-pregnancy to increase the transplacental transfer of copper to the lamb. This early and high dose of copper is essential as the placental transfer of copper from dam to foetus is relatively inefficient (Hendy & Evans, 1977).

Many different copper compounds have been tested and most rejected, often because of their potential to cause local tissue reaction (Harvey & Sutherland, 1953; Cunningham, 1959; Uvarov, 1970). Oral copper compounds vary in their absorption rates and in the faecal and urinary excretion rates (Lassitter & Bell, 1960). In general the absorption rate of oral copper is very low and repeated dosing requires considerable labour, which is expensive; factors which make its use unattractive (Fearn & Habel, 1961).

Copper glycinate (aminoacetate) at a dose rate of 45mg for ewes, effectively raises plasma and liver copper levels (Fearn & Habel, 1961; Hemingway et al., 1970); 80% of the available copper being stored in the liver (Camargo et al., 1962). The disadvantages of this material are that it causes various degrees of local necrosis (Harvey & Sutherland, 1953; Allcroft et al., 1959) and doses of 100mg have proven very toxic (Harvey, 1953): eight out of eleven sheep were killed by that dose in one experiment.

Copper methionate given as a 50mg dose to adult ewes was shown to raise plasma and liver copper levels (Hemingway et al., 1970; Whitelaw, 1980; Mahmoud & Ford, 1981; Suttle, 1981b; Mahmoud & Ford, 1982). The product has a slow uptake (Suttle, 1981b), and causes very severe local reactions (Camargo et al., 1962; Suttle, 1981b). On the other hand animals can tolerate a higher dose rate of copper methionate as, only 10-50% is retained in the liver (Camargo, 1962).

Copper oxyquinoline sulphonate, as a solution containing 6 mg Cu/ml, is rapidly translocated from the injection site (Suttle, 1981b; Mahmoud & Ford, 1982), and nearly 100% is retained in the liver (Cunningham, 1959). It causes minimal tissue reaction (Harvey & Sutherland, 1953) but causes toxicity at doses of 1-2mg per kilogram bodyweight (Mahmoud & Ford, 1981; Suttle, 1981b), and accordingly only relatively small total amounts of copper can be given in this form: the recommended dose is only 6mg for ewes (Bruere, 1980). Copper oxyquinoline sulphonate appears to afford less protection to sheep feeding on a diet high in molybdenum (Suttle, 1981b).

Copper edetate (versenate) has been the most favoured parenteral copper compound as it effectively raises plasma and liver copper levels (Harvey & Sutherland, 1953; Camargo et al., 1962; Hemingway et al., 1970; Suttle, 1981b; Mahmoud & Ford, 1982). This product has an intermediate translocation rate when compared to copper glycinate and copper oxyquinoline sulphonate (Suttle, 1982b) and causes minimal tissue damage at the site of injection (Harvey & Sutherland, 1953). It has caused deaths experimentally at dose rates of 3mg per kg (Mahmoud & Ford, 1982) and even at lower dose rates on some farms when used therapeutically (Harvey & Sutherland, 1953; Ishmael et al., 1969). However these losses have not exceeded 1 in 2,000, and only 3% of all flocks treated were affected (Ishmael et al., 1969; Hendy & Evans, 1977).

More recently copper oxide needles, which when given orally are retained in the abomasum, have been shown to increase plasma and liver copper concentrations both in lambs (Whitelaw, 1980) and in adult sheep (Ellis, 1981; Whitelaw, 1980; Suttle, 1981a; Judson et al., 1982). They have a liver copper retention of 8.3% (Suttle, 1981b). Due to the slow release of copper over 7-8 weeks (Dewey, 1977; Ellis, 1981) there is minimal risk of toxicity using doses up to 10gm and even when pretreatment liver copper concentrations exceed 1,000 ppm D.M. (Ellis, 1981).

Copper oxide suspended in olive oil and injected intramuscularly has been shown to raise plasma and liver copper concentrations (Lamand, 1978b) as has insoluble copper dust (Lamand, 1978a). Although both agents produce a moderate degree of inflammation, the fact that they are administered intramuscularly makes their use undesirable.

In 1980, Mallinson et al., and in 1982, Moore et al., experimented with controlled release glasses as a medium for copper therapeutic agents. These glasses are implanted subcutaneously and dissolve in from 1 to 15 days, depending on their composition. Up to 80% of the incorporated copper was retained in the liver and in addition plasma copper concentrations were raised. The glasses that dissolve over a longer period (5 days and greater), tend to produce a reaction at the site of implantation for up to 28 days after implantation. This medium for supplementation has promise as doses of 100 mg of copper can be given to sheep without any apparent indication of toxicity.

Copper toxicity in sheep

Sources of Copper

There are numerous reports of copper toxicity in sheep. These have been caused in many different ways because of the wide variety of sources from which copper may be derived.

Environmental contamination of soil and plant material occurs naturally in cupriferous soil outcrops. Contamination has also been caused by mine tailings and from corrosion of metallic copper. This contamination is the result of a high concentration of copper in the superficial soil layers as copper is not a mobile element (Tiller & Merry, 1981). Sheep have also been poisoned from ingesting pasture contaminated by copper which has been deposited from industrial pollution such as mine tailings (Lander, 1912; Bisset, 1934; Wiemann, 1939; Bischoff & Haun, 1939; Tiller & Merry, 1981).

Some plants, such as "skeleton weed" (Chondrilla juncea) (Bull, 1956), become potentially toxic because of their ability to accumulate high levels of copper (Skinner, 1960; Woolhouse & Walker, 1981). Legumes, such as clovers and lucerne, frequently have high concentrations of copper (up to 20 ppm D.M.), when growing in soils of high copper content (Bull, 1956; Robson & Reuter, 1981). Other plants, such as Senecio spp (ragwort) (Bull, 1956; Mortimer & White, 1975), Heliotropeum europeum (heliotrope) (Bull, 1956; Bull et al., 1956), lupin (Bennetts, 1957; Allen et al., 1970) and Echium spp (Paterson's curse) (St George et al., 1962; Connors, 1979), may indirectly cause copper poisoning due to the hepatotoxic action of their alkaloids. Such alkaloids can produce hepatic necrosis leading to the release of liver copper and the precipitation of a haemolytic crisis.

The application of copper to the soil has been responsible for copper toxicity. Possible sources are orchard sprays (Bordeaux mixture) (Faum & Seelinger, 1898; Schaper & Luetje, 1931; Beijers, 1932; Lafenetre et al., 1935; Fincham, 1945; Muth, 1952; Ogilvie, 1954), copper topdressing (Pryor, 1959; Tiller & Merry, 1981), pasture spraying of pig slurry (Loosmore, 1969; Batey et al., 1972; Feenstra & van Ulsen, 1973; Kneale & Howell, 1974; Dalgarno & Mills, 1975; Suttle & Price, 1976; Tiller & Merry, 1981), and of sewage sludge (Tiller & Merry, 1981), and copper sulphate which has been used as a molluscicide in liver fluke control (Gracey & Todd, 1960).

Housed animals have been poisoned frequently by copper. This has occurred when feed concentrates containing a high copper content, but often a low molybdenum content, have been fed continuously (Clegg, 1956; Pearson, 1956; Bracewell, 1958; Senior, 1959; Ross, 1964; Hill & Williams, 1965; Watt, 1966; Hogan et al., 1968; Adamson et al., 1969; Todd, 1969; Buck, 1970; Tait et al., 1971; Hartmanns, 1975; Arora et al., 1977; Bundza et al., 1982).

Ironically the use of copper therapeutically has also produced a number of animal deaths. Reports state that all these losses have been caused by the careless use of a wide range of copper containing products. These include copper sulphate-nicotine sulphate mixture as an anthelmintic (Boughton & Hardy, 1934), copper sulphate as a copper supplement (Boughton & Hardy, 1934; Eden, 1940; Naerland, 1948; Sharman, 1969), and copper chelates as copper edetate (Allcroft et al., 1965; Ishmael et al., 1969, Wiener & Field, 1970; Wiener & MacLeod, 1970) and copper oxyquinoline sulphonate (Cooper, pers. comm., Cunningham, 1959).

Some cases of copper toxicity have occurred when sheep have been moved on to pasture containing "normal" amounts of copper: for example, as in the seaweed eating North Ronaldsay (Wiener et al., 1977; MacLachlan & Johnston, 1982), and in Texel sheep eating normal concentrate feed (Arora et al., 1977).

The cases of copper toxicity in sheep confirmed in New Zealand by the Animal Health Laboratories since 1973, are set out in Table 1.3.

Clinical signs of copper toxicity

The signs of copper poisoning are dramatic and in most cases death follows rapidly. Animals affected with copper toxicity show depression and loss of appetite, which may continue for several days, but more often it lasts about 24 hours before the haemolytic crisis occurs. Once intravascular haemolysis begins, severe clinical signs develop and these include dyspnoea, increased heart rate, and haematuria; the latter being noticed in woolly sheep by the characteristic staining of the crutch. The mucous membranes become muddy-brown and the sheep may pass watery brown faeces. In most cases these symptoms result in death in 1-2 days. Occasionally sheep may recover from the haemolytic crisis, or they may die as a result of kidney damage or even from subsequent haemolytic crises.

Year	No. dead	Age	Source or predisposing cause
1973	several	9 mths	parenteral
1974	2 cases	ewes	orchard spray
	10/100	ewes	ragwort
	2	lambs	concentrate feed
1976	3 cases	ewes	copperised salt lick
			copperised superphosphate
1977	15/3000	ewes	ragwort & copperised superphosphate
1980	1 case	9 mths	penfed on concentrate
	1 case	lambs	copperised salt lick & pig mash
	1 case	9 mths	lucerne meal
1981	3/week over	9 mths	oral supplementation
	10 weeks		
1982	6/1200	lambs	oral copper & ewes supplemented

Table 1.3: Reported cases of copper toxicity in sheep in N.Z. taken from "Surveillance" reports of Animal Health Laboratories.

Post-mortem changes in copper toxicity

Animals which have died of copper toxicity show pathological changes which are typical of a haemolytic crisis. There is a marked jaundice throughout all tissues, and the mucous membranes often have a muddy-brown appearance. The liver is swollen, with rounded edges and is pale yellow in appearance. The kidneys often have a black metallic sheen, the so called "gun-metal" kidney. Ecchymotic and petechial haemorrhages are often seen between the muscle planes and subcutaneously. These changes are more evident in animals which have been handled or accidentally injured prior to death. Such post mortem changes appear consistently and have been reported by the many

workers in this field.

Although copper poisoning is often classified into acute and chronic forms, depending on the duration and cause of accumulation of copper by the body, there is only one main event which initiates poisoning. It is the accumulation of excessive amounts of copper in the lysosomes which causes release of the lysosomal contents and these initiate the extensive hepatic necrosis.

Ishmael et al. (1971a) have reported sudden deaths in sheep within 24 hours of copper administration. In these cases the condition is more typical of a heavy metal poisoning and it is characterised by excess fluid in the serous cavities, subendocardial haemorrhages, a congested abomasal mucosa, fluid intestinal contents and sometimes rectal haemorrhage. There is no generalised jaundice of the carcass. Histology of the liver reveals some centrilobular necrosis, but on chemical analysis liver copper levels are only 300-500 ppm D.M.

Changes in blood in copper toxicity

In copper poisoning the changes in blood parameters only occur near to the haemolytic crisis.

Blood copper concentrations rise very quickly. Within 48 hours they may have risen to ten times the normal concentration (Marston, 1952; Barden & Robertson, 1962; Todd & Thompson, 1963; Ishmael et al., 1972). However in some cases elevated blood copper concentrations have been recorded up to 28 days before the crisis (Sutter et al., 1958; McCosker, 1968; MacPherson & Hemingway, 1969). In other cases a mild "crisis" has probably occurred without visible haemolysis or haematuria although McCosker (1968) has reported a two-fold increase in blood copper concentration seven days prior to the crisis period.

Circulating copper ions appear to be the cause of haemolysis as the resolution of haemolysis parallels the fall in the blood copper concentrations (Ishmael et al., 1972). The increase in copper content of whole blood is much greater than the increase in copper levels in plasma. This is due to the pronounced increase in erythrocyte copper concentration (Ishmael et al., 1971a). The increase in plasma copper is due to an increase in copper ion concentration. Caeruloplasmin activity alters very little (Maribei, 1978), although Ishmael et al. (1972) have reported a two-fold increase in caeruloplasmin just prior to the haemolytic crisis.

An important prodromal feature of copper poisoning is the marked elevation of the packed cell volume (PCV), sometimes to as much as 55%, and occurring up to seven days in advance of the haemolytic crisis (McCosker, 1968; MacPherson & Hemingway, 1969; Ishmael et al., 1972). The dramatic increase may be caused either by erythrocytic enlargement or by dehydration. Once the haemolytic crisis becomes evident the PCV drops in direct relation to the severity of haemolysis. Should the animal survive, the PCV usually returns to normal concentrations over a ten day period.

During the period of intravascular haemolysis, methaemoglobin is present in the blood as evidenced by its characteristic chocolate-brown colour. Todd & Thompson (1961) found that this methaemoglobin is intracorpuscular and constitutes up to 35% of the total haemoglobin. As erythrocytes become lysed, the haemoglobin and methaemoglobin are released into the plasma. Soli & Froslic (1977) also demonstrated that the methaemoglobin formation during the terminal crisis is an intracorpuscular process and that it occurs before any osmotic fragility of red cells can be detected.

The first change detected before the onset of the haemolytic crisis is a fall in erythrocyte glutathione concentration. Two to four hours prior to the appearance of haemoglobin in the plasma there is a build up of Heinz bodies in the erythrocytes (from 1% in normal blood to 50% in pre-haemolytic blood) (Soli & Froslic, 1977) whereas

during the crisis proper, Heinz bodies appear in up to 90% of the erythrocytes and free Heinz bodies are seen in the plasma. Although the red blood cells maintain their osmotic status before the haemolytic crisis, they soon begin to lose shape and become distorted (Soli & Frosliie, 1977). Some erythrocytes are contracted or crenated and some appear as ghost cells devoid of haemoglobin but still contain Heinz bodies.

Reticulocytes and normoblasts do not appear in the peripheral blood until 20-24 hours after the commencement of the crisis. Leucocytes appear to be unaffected by copper toxicity although Soli & Frosliie (1976) have reported an increase in the number of monocytes within 24 hours of the onset of the crisis. There is an increase in neutrophils as cellular debris is released into the circulation during haemolysis.

Changes in liver in copper toxicity

Because the liver is the main storage organ for copper, its tissues are the first to be affected in copper poisoning. The changes that occur in the liver during the process of copper accumulation have been observed by several means. Histological changes have been monitored, and the "leakage" of hepatic enzymes into the circulation has been measured. It has been found that the activity of these enzymes are correlated with the concentration of liver copper, and blood copper, as well as to the total copper intake.

Centrilobular necrosis is the first observed histological change and may be seen within 48 hours following the administration of copper (Ishmael & Gopinath, 1972). As the liver copper concentrations increase, the zones of conspicuously altered cells widen and become extended towards the central veins (King & Bremner, 1979). Just prior to haemolysis most liver parenchymal cells appear to be severely affected (Ishmael et al., 1971b). Neutrophils start to invade the necrotic areas and there is centrilobular

congestion and haemorrhage. Rubeanic acid-positive granules are seen both in the cytoplasm of hepatic parenchymal cells and in swollen Kupffer cells as the copper content increases (Gooneratne et al., 1980). As the number of necrotic cells increases, the reticular framework in these areas of the liver starts to collapse (Ishmael et al., 1971b). At the time of haemolytic crisis there are further changes; such as the appearance of large foci of necrotic liver cells, often associated with foci of polymorphonuclear leucocytes. Eventually bile pigments start to accumulate in the canaliculi and numerous fat droplets are present in the viable cells (Ishmael et al., 1971b). The Kupffer cells appear large and numerous, and they contain eosinophilic and brown granular or globular cytoplasm, especially in the areas adjacent to the central vein (Gooneratne et al., 1980).

In sheep that survive the haemolytic crisis, cords of new parenchymal cells start to appear. Necrosis, polymorphonuclear infiltration and ballooning are no longer evident. These regenerative changes may be visible within nine days after copper administration (Ishmael & Gopinath, 1972) and sheep surviving up to three haemolytic crises may, after 40 days, show an increase in portal connective tissue. This may even extend into the periportal zone of the lobules (Gopinath & Howell, 1975).

King and Bremner (1979) saw evidence of apoptosis (Kerr et al., 1972) in the form of ovoid masses of condensed cytoplasm, nuclear remnants and other cellular debris. Apoptosis is a mechanism of controlled cell deletion involving the phagocytosis and degradation of these ovoid masses by other cells (Kerr et al., 1972).

Changes in liver enzymes in copper toxicity

Early tissue damage may often be detected by measuring the activity of cellular enzymes that have escaped from the damaged cell into the serum. Necrosis of hepatic cells or an alteration to the permeability of the cell wall will cause leakage of cellular

contents, in particular cellular enzymes (Kramer, 1980). Several enzymes are found predominantly in hepatic cells. Although they may be present in other tissues their levels in those tissues are insignificant.

These enzymes can be monitored in serum. It has been found that the quantity of enzyme present in serum bears a relationship to the amount of liver damage. The increase in serum enzyme activity is dependent upon three factors. These are the enzyme measured, the half life of that enzyme in serum, and the molecular size of the enzyme. The amount of alteration in cell wall permeability determines the amount of enzyme entering the blood stream. The smaller the molecular size of the enzyme the more readily it will permeate the cell wall.

Table 1.4 gives a summary of specific liver enzyme analyses that have been used by workers in this field.

Changes in kidney in copper toxicity

The histopathological changes in the kidneys of sheep poisoned by copper are seen mainly as degenerative changes in the proximal convoluted tubules and indicated by epithelial necrosis, desquamation and vacuolation (Ishmael et al., 1971b). The tubular lumina contain a granular eosinophilic material (Soli & Nafstad, 1976) which is positive to rubeanic acid and Perl's iron stain (Gopinath & Howell, 1975).

During the pre-haemolytic phase of copper toxicity when copper is accumulating in the tissues, the only discernible histological change is the presence of eosinophilic intracytoplasmic granules in the epithelium of the proximal convoluted tubules. The number and size of these granules is dependent upon the duration of exposure to copper excess (Gopinath et al., 1974).

	SGOT	SDH	GD	LDH	arg
Todd & Thompson(1963)	*			*	
Ross(1964)	*				
van Adrichem(1965)	*			*	
Ross(1966)	*				*
MacPherson & Hemingway(1969)	*				
Ishmael,Gopinath & Treeby(1971)	*	*	*		*
Ishmael,Gopinath & Howell(1971)	*	*	*		*
Ishmael & Gopinath(1972)	*	*	*		
Ishmael <u>et al.</u> (1972)	*	*	*		*
Todd & Thompson(1973)	*			*	
Thompson & Todd(1974)	*				
Gopinath & Howell(1975)	*	*	*		
Bath(1979)	*				
Buckley & Tait(1981)	*				

SGOT = Serum glutamate oxaloacetate transaminase
 SDH = Sorbitol dehydrogenase
 GD = Glutamate dehydrogenase
 LDH = Lactate dehydrogenase
 arg = Arginase

Table 1.4: List of workers and the serum enzyme analyses they have used in the investigation of copper toxicity.

Once the haemolytic crisis occurs, the cells of the proximal convoluted tubules show increased eosinophilic and intracytoplasmic granules, many of which stain positively for haemoglobin, copper and iron. The cortical tubules become dilated and contain many eosinophilic and granular casts (Gopinath et al., 1974).

In the post-haemolytic phase the same histological changes are still evident. During the accumulation of copper in the kidney it is thought that the increase in the size and number of intracytoplasmic granules is a lysosomal response to the increased copper storage. The sequestration of copper into the lysosomes protects the remainder of the cell from the toxic effects of the metal (Goldfischer et al., 1970).

It is believed that the accumulation of numerous casts of haemoglobin in the kidney tubules cause an impairment of function that produces the renal failure in copper toxicity.

Changes in brain in copper toxicity

The histopathological changes in the tissues of the central nervous system of sheep which have died from copper poisoning, have been examined and reported upon by Doherty et al., (1969), Ishmael et al., (1971c), Hooper (1972), Morgan (1973), Howell et al., (1974), Gopinath & Howell (1975), and Gooneratne & Howell (1979).

Doherty et al., (1969), described the lesions seen in the brains of six sheep poisoned by copper sulphate, as a marked spongy transformation predominantly in the white matter, especially the midbrain, pons and cerebellum. Ishmael et al., (1971c), confirmed these findings.

Hooper, (1972), described this spongy degeneration as the occurrence of large empty vacuoles in or along myelin sheaths in the white matter; and in single axons traversing adjacent grey matter of the brain and spinal cord. The brain stem was commonly and severely affected. He called this condition status spongiosus and compared it to the changes seen in ruminants poisoned by hepatotoxic pyrrolizidine alkaloids of plants and other hepatic diseases such as severe liver fluke infestation, ischaemic liver necrosis, facial eczema, and heart failure associated with liver necrosis.

Using electron microscopy Morgan, (1973), found that the clear spaces around the large myelinated axons were intramyelinic vacuoles produced by the separation of lamellae at the intraperiod line. He found that the oligodendroglia, neurones and their processes and blood vessels were apparently normal as were the axons within the severely distended myelin sheaths.

Howell et al., (1974), produced vacuolation in the myelin sheaths of 12 out of 20 sheep that either died of copper toxicity, or were killed during the haemolytic crisis. Ultrastructural studies revealed that the vacuoles associated with the nerve fibres were in the outer tongue of oligodendrocyte cytoplasm and the swollen astrocytes contained more glycogen, mitochondria and endoplasmic reticulum than normal. They concluded that the changes in the central nervous system due to copper poisoning were the result of the effects of altered metabolic processes on glial transport mechanisms.

The brains of six out of seven copper-poisoned sheep examined by Gopinath and Howell, (1975), showed lesions varying from swelling of the myelin sheath in the medulla, cerebellum, and dorso-lateral area of the thalamus, to pronounced vacuolation of white matter in all sections of the brain.

The levels of copper, iron and zinc in brains of normal sheep were compared with those from sheep which had been poisoned with copper (Gooneratne and Howell, 1979). Surprisingly, there was no measureable increase in copper or the other two elements in sheep which had died from copper poisoning. As claimed by Morgan, (1973), and by Howell et al., (1974), it is likely that the degenerative changes which occur in the brain of sheep poisoned with copper are terminal and due to other toxins released as a result of liver damage.

Hooper et al., (1974), poisoned fourteen sheep with the hepatotoxic alkaloid lasiocarpine. All sheep developed severe hepatic necrosis: seven of these sheep developed spongy degeneration

of the central nervous system. They found a positive correlation between the appearance of spongy degeneration and the development of elevated blood levels of ammonia and of glutamine in the cerebro-spinal fluid. This further showed that status spongiosus is a terminal effect associated with severe liver necrosis.

Changes in muscle in copper toxicity

Muscle changes have been studied by Thompson and Todd, (1974), and by Gooneratne and Howell, (1980). There appear to be no detectable histological changes in the muscle tissue but during the period of haemolytic crisis there is a rapid rise in creatine phosphokinase blood levels which in the sheep that survive the haemolytic crisis return to normal in about three days.

The increase in serum enzyme activity appears to be the result of cellular enzyme escaping through the cell membrane. This increase in cell membrane permeability also occurs in hepatocytes, erythrocytes, and cells in the kidney and brain. The suggested causes are those of hypoxia, hypercupraemia and possibly a decrease of selenium and or vitamin E content in the blood and muscle tissues.

CHAPTER 2. ESTABLISHING BIOCHEMICAL PARAMETERS TO MONITOR THE
EFFECTS OF EXCESSIVE COPPER USED THERAPEUTICALLY

INTRODUCTION

Copper preparations that are intended for parenteral administration in sheep are often used to prevent or to treat copper deficiency. Certain of these preparations under special circumstances have caused deaths. In order to evaluate the effects of copper therapy on the animal, a number of different biochemical parameters were measured. The alteration in the biochemical parameters should indicate the earliest signs of impending toxicity.

In this experiment repeated doses of a copper therapeutic agent were administered to sheep, while the appropriate biochemical parameters were under constant surveillance. Supplementation with copper was continued until each sheep underwent a haemolytic crisis. The copper compound used in this experiment was copper calcium edetate as it is a chelate with a medium translocation rate when compared to other copper salts (Suttle, 1981b).

The parameters used in this study included liver copper concentrations, serum levels of liver enzyme activity, histopathology of liver tissue, and haematological changes. The liver is the main storage organ for copper, and regular determination of the liver copper concentration will indicate the changes in copper status of the animal. Because hepatotoxic damage occurs in copper toxicity and because the hepatocellular enzymes pass out of the damaged hepatocytes and into the plasma, changes in the levels of the two liver-specific enzymes, serum glutamate oxaloacetate transaminase (SGOT) (EC 2.6.1.1) and sorbitol dehydrogenase (SDH) (EC 1.1.1.14) should indicate early changes in liver cells. SGOT is the conventional enzyme used to assess liver damage, while SDH is liver specific and has a short half-life of 36 - 48 hours. (Traces of SDH may be found in the testis and retina but are inconsequential).

Histopathological examination of liver may help to demonstrate when liver damage has taken place. Alteration in the conventional blood parameters such as packed cell volume, haemoglobin, plasma protein, and the icteric index, are indicative of any haemolytic changes, while changes in the blood cells may provide some indication of the body's response to any toxic effects caused by the excess copper.

MATERIALS AND METHODS

Two eighteen month-old Perendale rams were used initially to determine whether the taking of a liver biopsy under general anaesthesia, using sodium pentobarbitone¹, might cause changes in the levels of SGOT and SDH activity in the serum. Enzyme activity levels in the sera were estimated prior to the liver biopsy and then twice daily for seven days.

The results showed a slight rise above normal in enzyme activity at between 24 and 36 hours, but these levels had returned to normal within 48 hours. It was concluded that the effects of liver biopsy would be insignificant at 72 hours.

On completion of this initial investigation these same two rams, maintained on a hay diet containing 1.67 ppm D.M. of copper, were injected once a week with 50mg of copper as copper calcium edetate contained in a 2ml dose². The injections were given subcutaneously in the dorsal region of the neck. The sites chosen in the neck were the anterior and posterior dorsal areas, and they alternated between left and right sides. Blood and serum samples were collected from the jugular vein immediately before the injection and at 12, 24, 36, 48, and 96 hours after the injection.

Every second week, on the fifth day after the copper injection, each sheep was anaesthetised with sodium pentobarbitone. Liver biopsy samples were taken by aspiration from the diaphragmatic surface of

¹ Anathal, V.R. Laboratories (Aust.) Pty. Ltd.

² Coprin Multidose, Glaxo (N.Z.) Ltd.

the dorsal lobe of the liver. The liver biopsy technique used was based on the aspiration technique developed by Dick (1944), but modified so that the liver was entered from the diaphragmatic surface. The point of entry for the biopsy probe was at the right paralumbar fossa approximately three centimetres posterior to the angle of the last rib (Figure 2.1). Each liver sample was divided into two aliquots. One sample was immediately frozen at -4°C for later chemical analysis and the other sample was placed in 10% buffered formol saline for subsequent histopathological examination.



Figure 2.1: Path of the liver biopsy probe shown using a dissected specimen

As the blood samples from each sheep were collected, the injection sites of the copper calcium edetate were palpated to assess any tissue reaction and this was recorded. At the time of each liver biopsy both sheep were weighed.

The copper calcium edetate injections continued to be given weekly until signs of chronic copper poisoning appeared, or death occurred. The signs of copper poisoning were listlessness, hyperpnoea, haematuria, and yellow grey mucous membranes.

The "preliminary investigation" showed that the procedures outlined would allow the appropriate measurement of any changes to the animal's body in response to repeated copper injections. A full experiment was set up, utilizing a further nine eighteen-month old Romney rams. These rams were grazed on pasture containing on average 3.66 ppm D.M. of copper. Pasture samples were analysed for copper content monthly. After an initial liver biopsy, each ram was injected with 100mg of copper as copper calcium edetate contained in 4 ml, and then each subsequent week with a further 50mg of copper contained in 2ml of the same product.

Blood and serum samples were collected for the measurement of the appropriate enzyme activities and blood parameters immediately before and at 48 and 96 hours after injection. Also at each bleeding, the injection site was palpated and any reaction recorded. Every second week liver biopsy samples were obtained for chemical analysis and for histopathological examination. Wool samples were also retained after shaving the biopsy site, and on each occasion the rams were weighed.

The Coprin injections were given weekly until the signs of copper poisoning developed, or death occurred. All rams that died were autopsied. Samples of liver, kidney, and brain were collected into 10% buffered formol saline for histopathological examination and aliquots of the same tissues were deep frozen to await chemical analysis.

The three rams that developed a haemolytic crisis but survived, were examined and sampled three times weekly until liver enzyme concentrations in the serum had returned to the pre-injection concentrations.

Blood analysis

All blood samples were analysed for haemoglobin content by the cyanomethaemoglobin method (Schalm *et al.*, 1975) and the packed cell volume of each was estimated by the haematocrit method (Schalm *et al.*, 1975). In each case the mean corpuscular haemoglobin content (MCHC) was calculated from the former two readings. The plasma protein content was determined using a refractometer (Schalm *et al.*, 1975), and the icterus index was determined by comparing the colour of the plasma with potassium dichromate standards (Schalm *et al.*, 1975).

Each whole blood sample was oxidised with hydrogen peroxide, digested with concentrated nitric acid, and taken up in 2N hydrochloric acid using a method devised by the author (Appendix 1). Inductively-coupled plasma atomic emission spectrometry (Lee, 1981), was used to determine the amounts of copper, iron, zinc, manganese, sodium, potassium, magnesium, calcium, phosphorus and sulphur present in each blood sample.

Approximately once a week a total leucocyte count was performed on a sample from each animal using isotonic saline as a diluent and a leucocyte counter³. Blood smears from this same sample were stained with Wright's stain and a differential leucocyte count was carried out.

While sheep 237 was undergoing a haemolytic crisis, plasma samples were collected on a serial basis and analysed for haemoglobin content using benzidine by the Crosby and Furth's modification of King and Barker's method (Dacie & Lewis, 1970). Erythrocytes from sheep 237 were collected in phosphate buffer in glutaraldehyde before and during the haemolytic crisis and subsequently processed for electron microscopy (Jain & Kono, 1972). As sheep 237 recovered from the haemolytic crisis, reticulocyte counts were performed using new methylene blue stain.

³ Cell-Crit 920-A Cell Counter, Royco Instruments Inc.

Liver enzyme measurement

Within two hours of collection the serum levels of SGOT and SDH were estimated. SGOT was measured using the method of the German Clinical Chemistry Association (Merck Test), and the SDH was measured using the Gerlach method (Boehringer Mannheim). Each enzyme assay was determined at 340nm wavelength using a spectrophotometer⁴.

Liver analysis

Liver biopsy samples were analysed chemically using a nitric acid digestion on ashed material. The digested material was taken up in 2N hydrochloric acid and the mineral content determined by inductively-coupled plasma atomic emission spectrometry (Appendix 2). The samples were analysed for the elements of copper, iron, zinc, manganese, sodium, potassium, magnesium, calcium, phosphorus, and sulphur.

Samples fixed in formol saline were processed, sectioned and stained. One section was stained with haematoxylin and eosin (H & E), and the other section was stained with rubeanic acid (r. a.)(Appendix 3).

Processing of tissues collected at post-mortem

Sections of tissues were ashed and digested in nitric acid preparatory to analysis for copper, iron, zinc, manganese, sodium, potassium, magnesium, calcium, phosphorus, and sulphur.

Formalised samples of liver and kidney were processed, sectioned and stained. One section of each was stained with H & E and the other section of each was stained with rubeanic acid (Appendix 3). In addition, kidney sections were stained with Perl's iron for iron pigment of haemoglobin. Brain sections were taken from the medulla, the cerebrum, the pons midbrain area, and the cerebellum. One series

⁴ Pye Unicam SP6-550 UV/VIS Spectrophotometer

of sections were stained with H & E, and another series of sections stained with luxol fast blue for myelin changes.

RESULTS

Blood

Prior to the onset of the haemolytic crisis the blood parameters were relatively stable. As the haemolytic crisis developed the PCV dropped rapidly as did the haemoglobin content and blood iron content. However during times of high liver damage, indicated by the elevation of liver enzyme levels, the MCHC rose above 40% and the icteric index rose, indicating that intravascular haemolysis had occurred (Figure 2.2).

The white blood cell counts remained within the normal range. There was a neutrophilia and later an eosinophilia in those sheep which survived the haemolytic crisis. Ten days after the onset of the haemolytic crisis in those sheep that survived, there was a reticulocytosis; indicating that the regeneration of erythrocytes was not impaired.

Chemical analyses of the blood showed no significant variation in the concentration of zinc, manganese, sodium, potassium, calcium, phosphorus, sulphur and magnesium. Iron levels fell at the same rate as the haemoglobin content of the blood.

The copper content of the blood tended to rise 12-48 hours after each injection of copper calcium edetate but returned to a normal level within four days. After the initial injection the blood copper concentration increased by 0.5 ppm. Following subsequent injections a smaller increase was measured. Within the 24 hours preceding the first signs of haematuria the blood copper levels rose rapidly from two to seven times normal levels (1.3 to 8.9 ppm). In the three surviving sheep, the copper levels returned to normal within seven days after the appearance of the haemolysis.

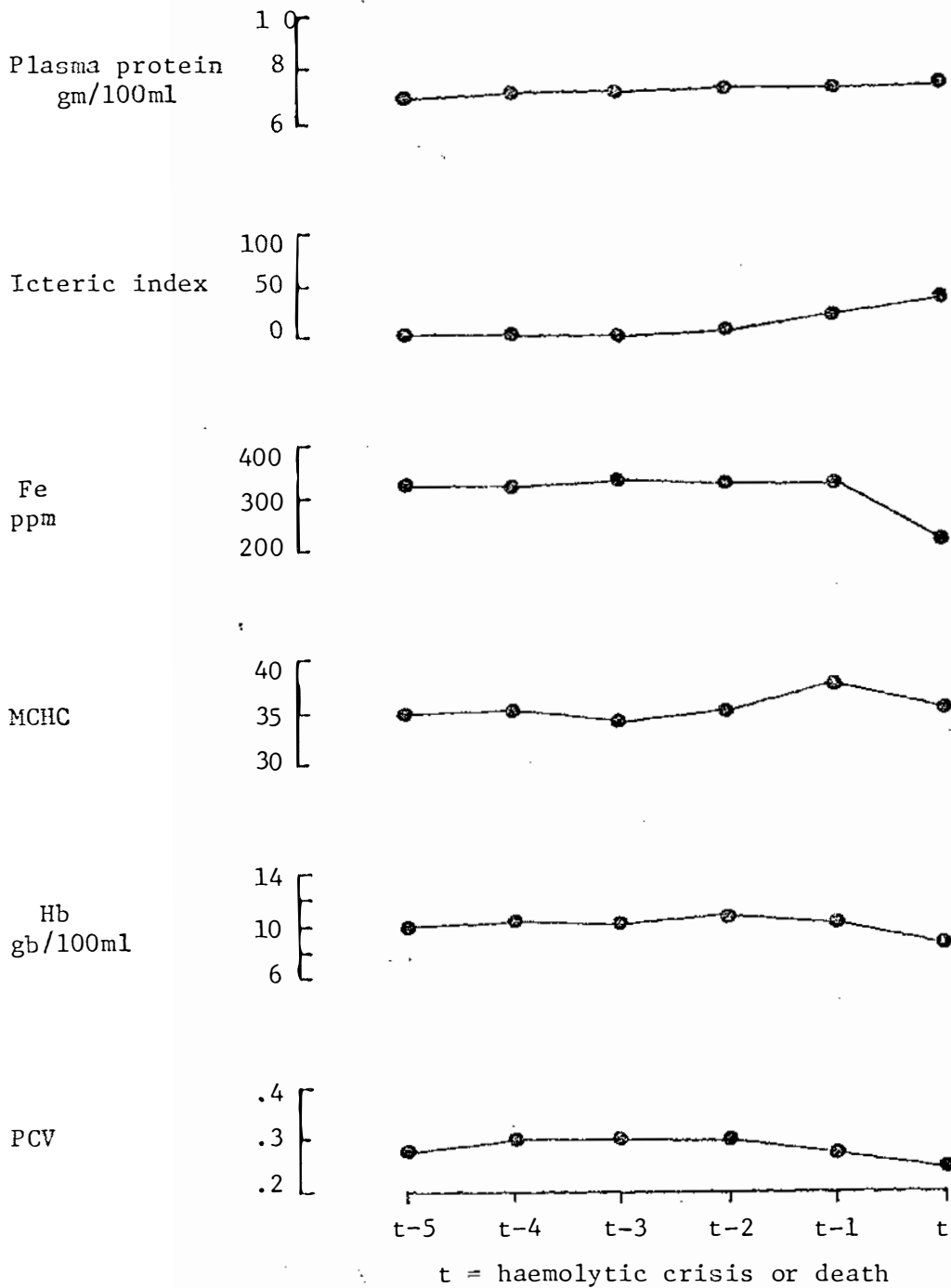


Figure 2.2: Average blood parameters of all sheep recorded from six daily bleedings prior to haemolytic crisis or death

Liver enzyme measurement

In some sheep there was a continued increase in the level of enzyme activity, and in other sheep enzyme activity reached a high peak and then showed very little change following subsequent copper injections. In all sheep SDH levels increased before the levels of SGOT rose, sometimes by as much as four weeks beforehand (Figures 2.3 & 2.4).

Liver digestion

Following injection with copper calcium edetate the chemical analysis of the liver showed an average increase of 245 ppm D.M. of copper for each 50 mg equivalent dose. Assuming the liver is 1.13% of the total bodyweight of the sheep, and the dry weight is 29.4% of the total wet tissue (van Ryssen, 1980); approximately 81.4% of each total dose of copper was retained in the liver. However at death, the liver copper content was always lower than the copper level measured at the time of the previous liver biopsy. The average loss of copper during this period was 780 ppm D.M. which was approximately 34% of total liver copper.

Those sheep which survived their haemolytic crisis and received no further copper supplementation also showed a decline in liver copper content over the ensuing two to three months. The decline was equivalent to a daily loss of approximately 0.66% of the initial total liver copper as measured at the time of the haemolytic crisis.

The chemical analyses of the kidneys collected at the time of autopsy are shown in Table 2.1. Also shown in this table is a summary of the liver chemical analyses and the results of the histopathology of the liver and kidney. The analyses of 27 brain sections for copper content averaged 24.0 (range 10 - 41) ppm D.M., and was not significantly higher than the copper content of brain tissue analysed from sheep of normal copper status. The

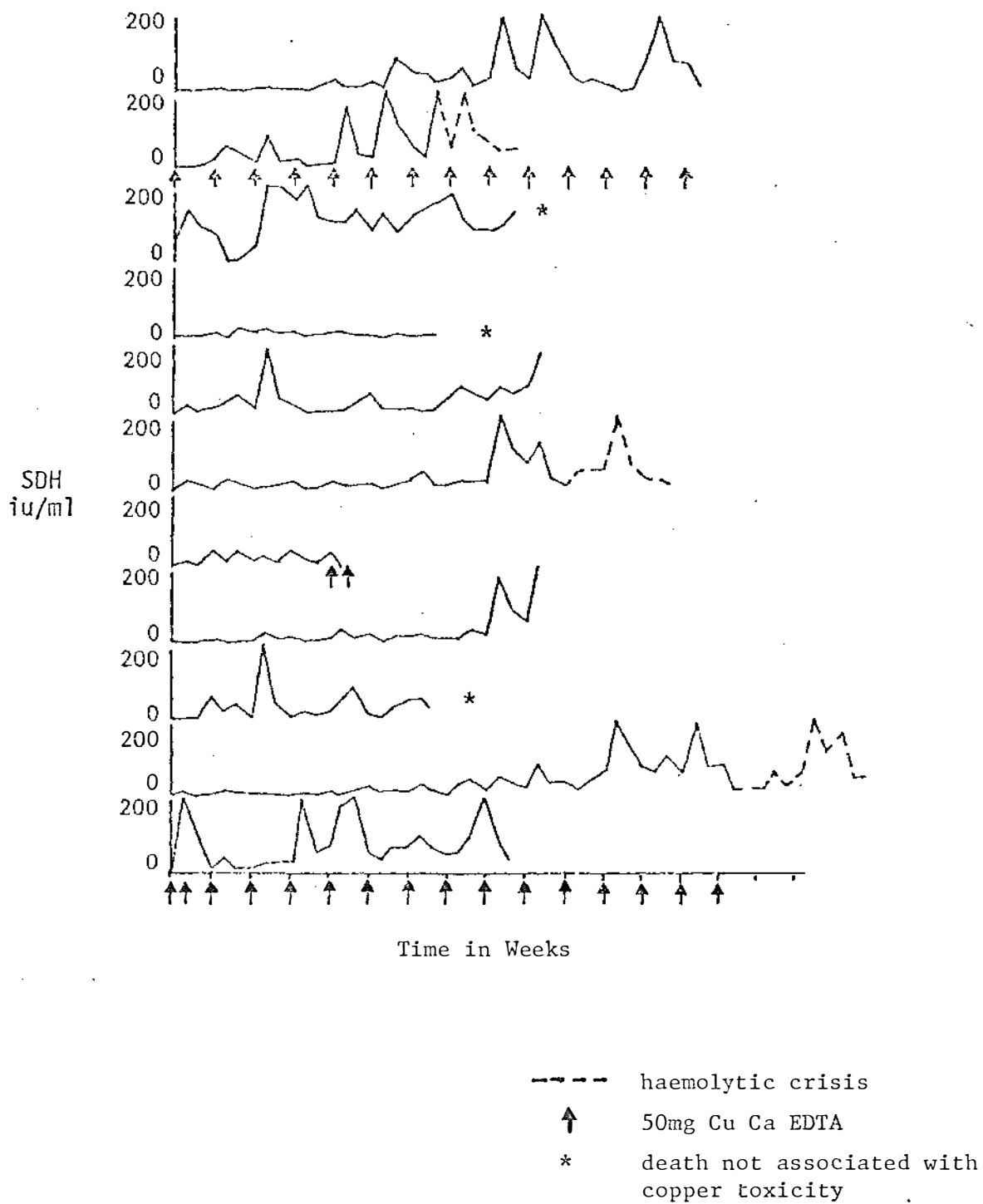


Figure 2.3: Changes in the sorbitol dehydrogenase activity in serum of eleven sheep subjected to weekly injections of copper calcium edetate

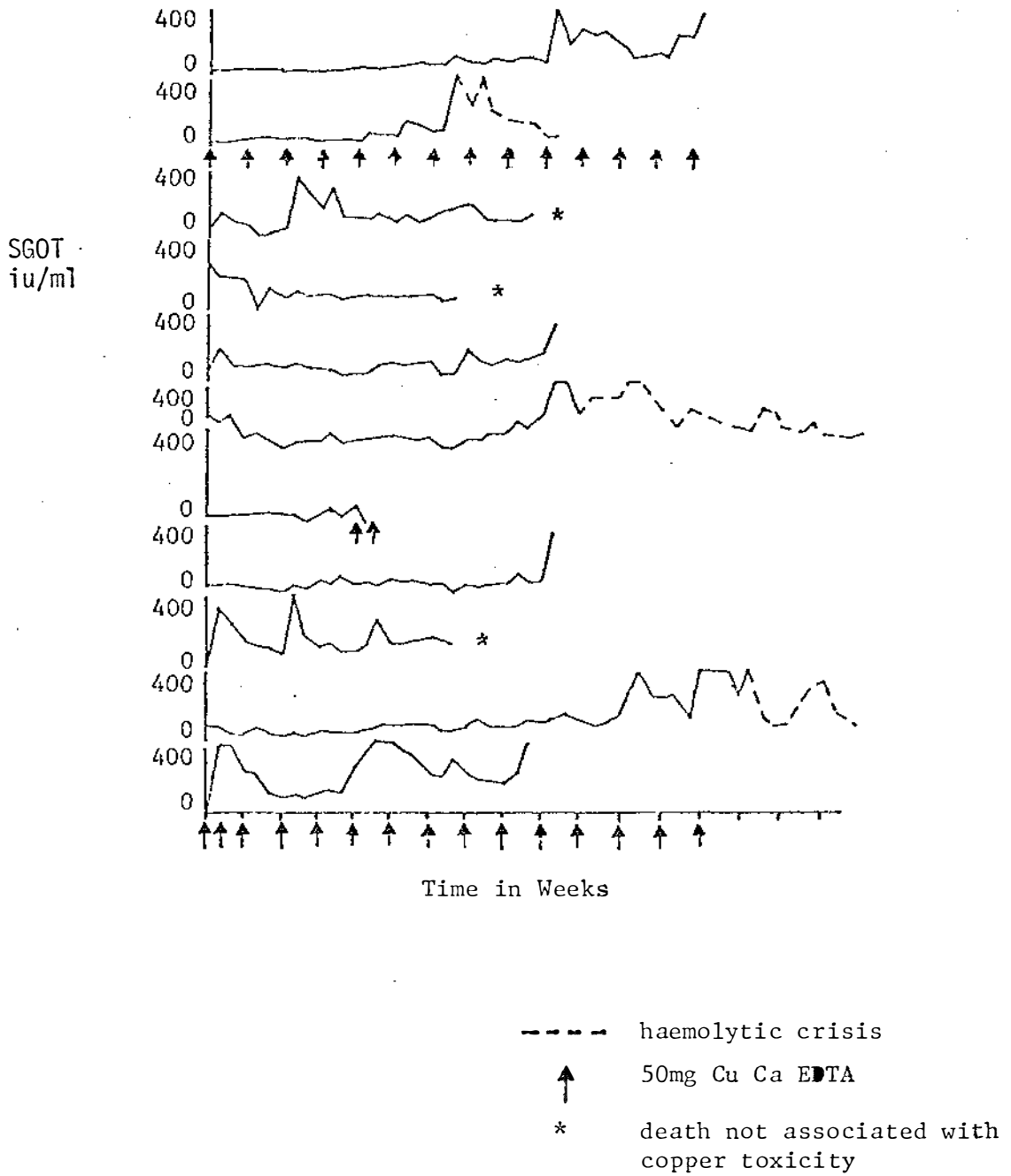


Figure 2.1: Changes in glutamate oxaloacetate transaminase activity in serum of eleven sheep subjected to weekly injections of copper calcium edetate

Sheep	Total Cu doses	Liver score	Copper content in ppm			Kidney score	Cause of death or
	no.each50mg	H&E	Liver predeath	Liver at death	Kidney	H&E	haem. crisis
237	7	5	2453	1931			haem. crisis
238	14	5	3070	2721	275	4	chronic tox.
12	10	5	2204	1962	416	5	chronic tox.
13	16	5	2774	2868			haem. crisis
14	8	3	2367	1267	26	1	not copper
15	11	5	2808	2370	1242	4	peracute tox.
16	7	5	1404	1178	186	5	chronic tox.
17	11	5	3123	2465			haem. crisis
18	11	5	2785	1967	978	4	peracute tox.
19	8	3	2061	1102	31	1	not copper
20	10	5	4448	2384	85	2	not copper

Histology key:

Liver H & E

1. No significant findings
2. Occasional macrophages & regular nuclei
3. Necrotic cells present
4. Pyknosis & necrosis, & aggregations of macrophages
5. 50% of nuclei enlarged, vacuolation present & aggregations of macrophages

Kidney H & E

1. Some protein in proximal tubules
2. Some necrosis & vacuolation in cells of proximal tubules
3. Pyknosis & necrosis in proximal tubules & macrophages present
4. Pyknosis and necrosis of tubules & macrophages infiltrating
5. Most tubules necrotic or distended, some pyknosis and invading macrophages

Table 2.1: Results of copper analysis and histology of tissues collected from sheep used in copper toxicity studies.

concentrations of the other elements in the sheep that died from copper poisoning and in the sheep that died from other causes were not significantly different.



(For tube numbers please see Table 2.2: page 49)

Figure 2.5: Serum from sheep no. 237 collected during the haemolytic crisis

The results of the blood collected from sheep no. 237 during the haemolytic crisis are shown in Table 2.2 and illustrated in Figure 2.5.

The crenation and formation of Heinz bodies within the erythrocyte is shown in the electron micrographs (Figures 2.6 & 2.7).

Analysis of wool samples

The results of the analysis of the wool samples collected are discussed in Chapter 7.

Day	Time	Tube No.	PCV	Blood Hb gm/100ml	MCHC Blood	Plasma Hb gm/100ml	gms Hb in rbc's	MCHC intact rbc's	%cells releasing Hb
1	pm	1	.24	8.6	35.8	.029	8.578	35.7	.26
2	* am	2	.22	7.8	35.5	.183	7.657	34.8	1.87
3	am	3	.18	7.1	39.4	1.003	6.278	34.9	13.09
3	pm	4	.16	6.6	41.2	1.010	5.752	36.0	14.74
4	am	5	.15	6.1	40.7	1.238	5.048	33.7	20.84
4	pm	6	.13	4.8	36.9	.753	4.145	31.9	15.80
7	am	7	.16	5.4	33.7	.045	5.362	33.5	.71
8	am	8	.17	5.8	34.1	.009	5.793	34.1	.12

* = start of haemolysis

Table 2.2: Changes in blood parameters of sheep no. 237 during a haemolytic crisis

Gross pathology

Five of the sheep died as a result of copper poisoning: three sheep showed signs typical of chronic copper poisoning, and the other two showed signs of acute copper poisoning. Three other sheep also died in this experiment, but the causes of death could not be attributed to copper toxicity.

The three sheep that died of chronic copper poisoning all developed jaundice (Figure 2.8) and showed petechial haemorrhages throughout the musculature (Figure 2.9). They had myocardial petechiae and blood-stained pericardial fluid: one sheep had 100 ml of serofibrinous fluid in the pericardial sac. Another sheep had haemorrhaged profusely from the nostrils and from the rectum. In each sheep the kidneys were enlarged and exhibited the typical blue-black gun-metal colour (Figure 2.10). The urine was the port-wine colour indicative of haemoglobinuria. In each sheep the liver was enlarged, pale and bronzed, and the edges were swollen.

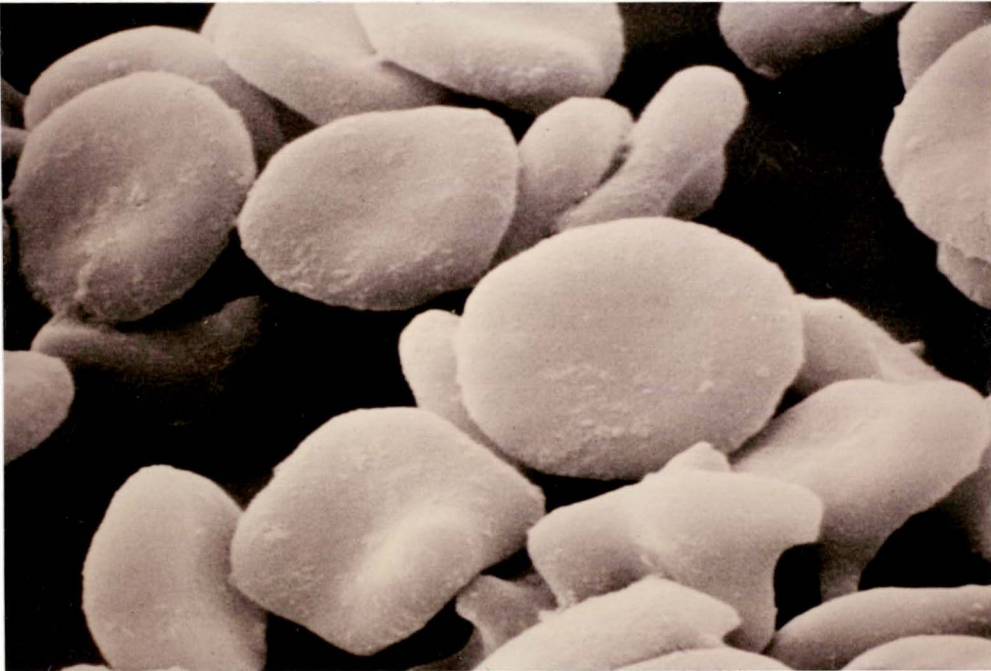


Figure 2.6: Electronmicrograph of normal erythrocytes
(8000 X magnification)

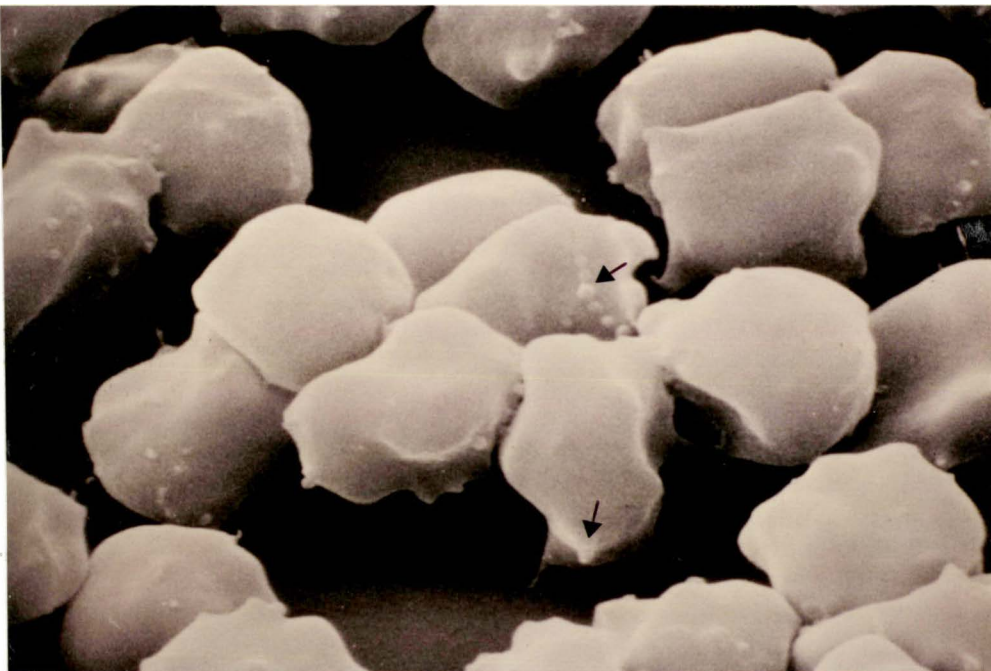


Figure 2.7: Electronmicrograph of erythrocytes taken from sheep
no. 237 during the haemolytic crisis. Note crenation and
Heinz bodies (A) (8000 X magnification)



Figure 2.8: Jaundiced conjunctival membranes in a sheep that died from copper toxicity



Figure 2.9: Petechial haemorrhages in the pectoral muscle planes of a sheep that died from copper toxicity

The two sheep that died of acute copper poisoning both showed similar post-mortem findings, but the carcasses were not jaundiced and there was no evidence of haemoglobinuria. The thoracic cavity was filled with 300-600 ml of a yellow/green translucent fluid containing some fibrin clots. There was also excessive fluid in the pericardial sac. The abomasal contents were fluid and in one sheep the kidneys were darkened.

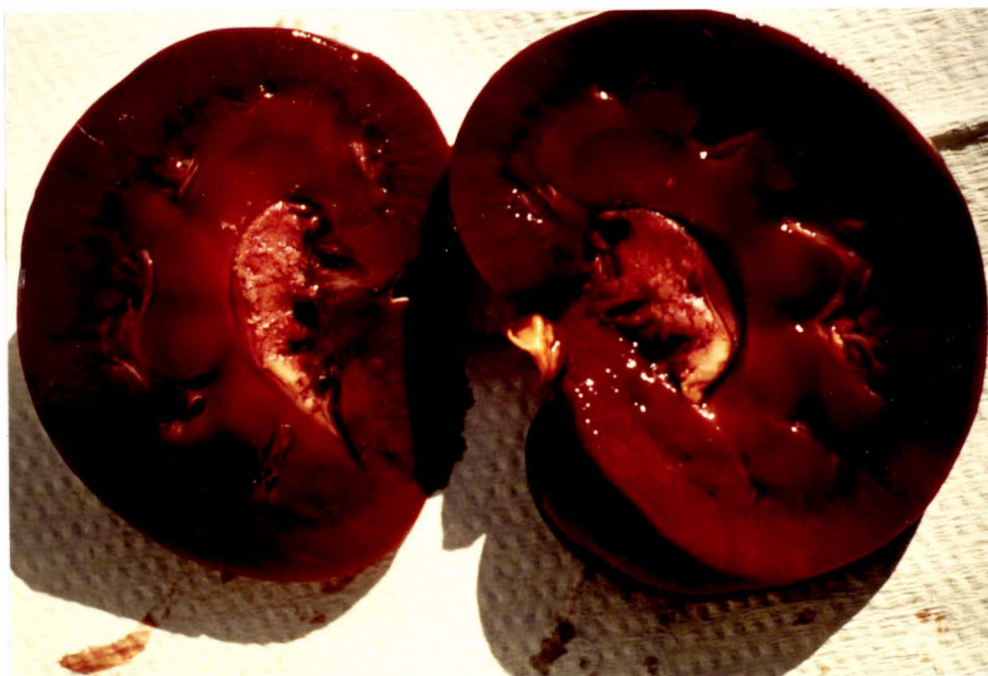


Figure 2.10: Typical 'gun-metal' kidneys from a sheep that died from copper toxicity

Histopathology

Liver tissue

Examination of liver sections taken prior to the first injection of copper calcium edetate, showed a normal liver structure. No copper granules were seen in those sections stained with rubeanic acid.

The histopathological changes in the liver of each sheep showed a consistent pattern. This pattern of change occurred progressively, and appeared to follow as a response to each additional dose of copper given to each sheep. In each case the first noticeable change was the appearance of occasional macrophages in the centrilobular area of the hepatic lobule. This was followed by the appearance of necrotic cells and swollen hepatocytes with pyknotic nuclei. Eventually the loss of the regular cording structure of the hepatocytes was seen and polymorphonuclear leukocytes then began to aggregate about the more extensive necrotic foci. Sections taken from dead animals or from animals during the haemolytic crisis showed at least 50% of hepatocytes degenerating, with pyknotic nuclei and vacuolated cytoplasm. There were large aggregations of macrophages surrounding these necrotic areas (Figures 2.11 & 2.12).

Sections treated with rubeanic acid stain showed a consistent and gradual increase in the greenish-black granules of copper. Initially there were occasional small granules in the cytoplasm of some of the centrilobular hepatocytes. These granules increased in size and number until approximately 30% of the centrilobular cells were so affected at death. During the haemolytic crisis, large aggregations of granules were seen in the cytoplasm of more than 50% of the centrilobular hepatocytes. These granules were also present in the cells adjacent to the central vein and portal triad, and also in the cytoplasm of the enlarged Kupffer cells (Figure 2.13).

Liver sections from those sheep which survived the haemolytic crisis, showed a gradual resolution of the cellular damage and a reduction in the size and number of greenish-black granules. Approximately 1% of the cells showed mitotic activity. This change was not evident before the haemolytic crisis but was present afterwards and indicated liver tissue resolution. The reduction in granules occurred first in the centrilobular area. The last area to show a reduction in the number of copper granules was the area adjacent to the central vein. Complete resolution was apparent in the liver of these sheep two months after the haemolytic crisis.

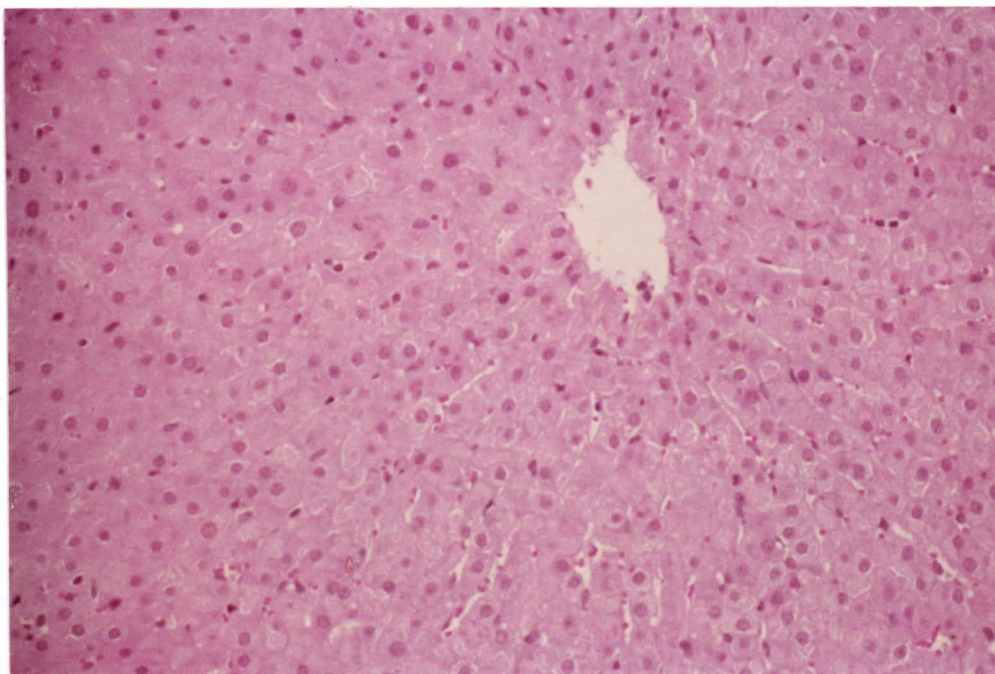


Figure 2.11: Histology of normal liver tissue (H. & E. stain)
(200 X magnification)

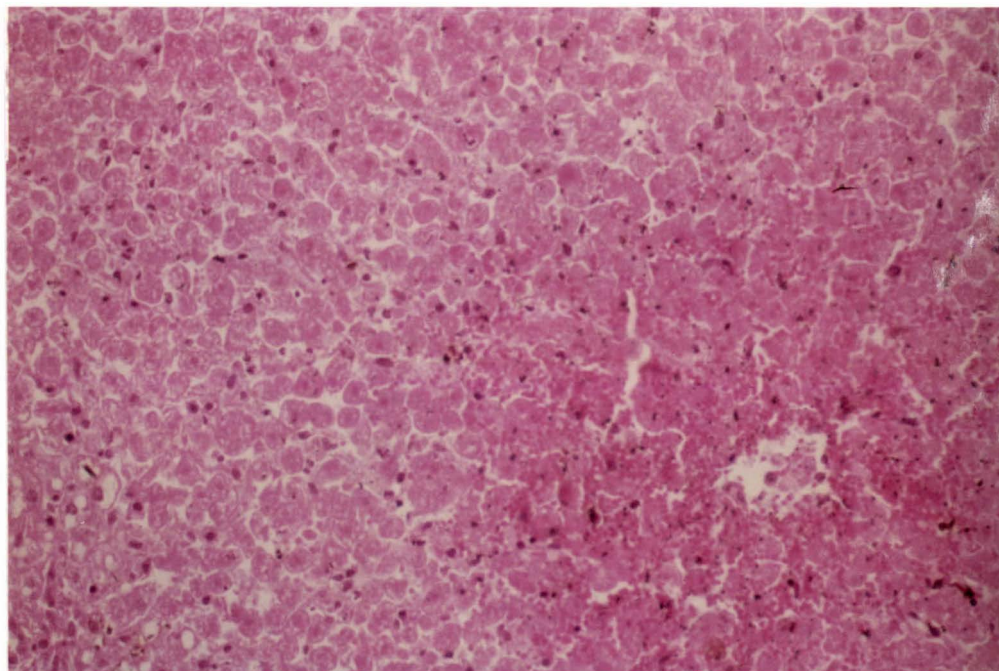


Figure 2.12: Histology of liver from a sheep that died
from copper toxicity (H. & E. stain) (200 X magnification)

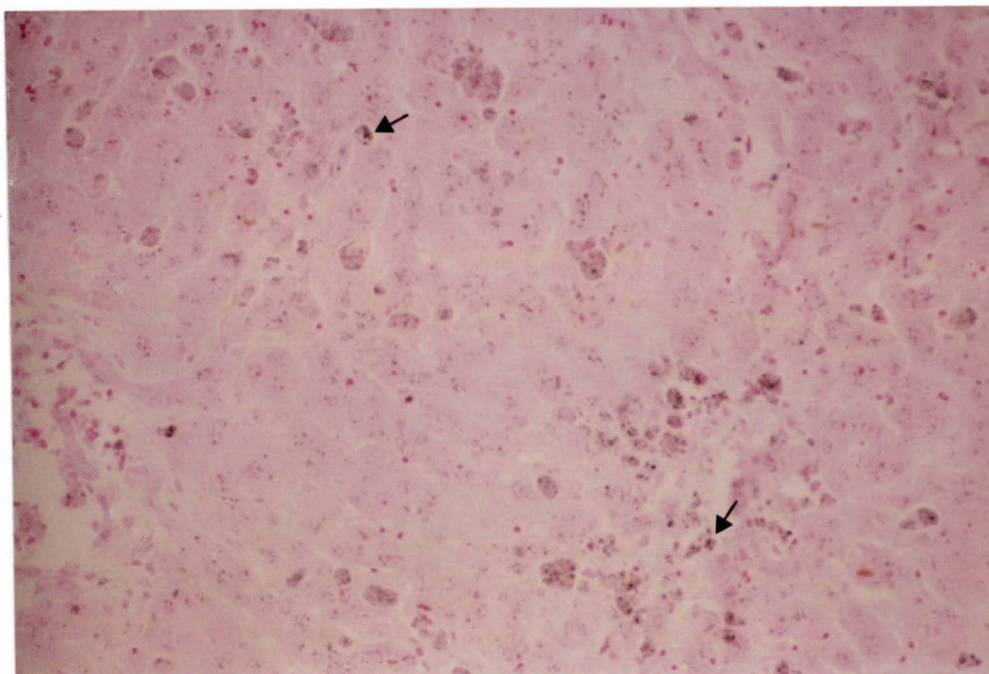


Figure 2.13: Histology of liver from a sheep that died from copper toxicity, showing greenish/black granules of copper (↑) (rubeanic acid stain) (200 X magnification)

Kidney tissue

The five sheep that died from copper poisoning all had similar lesions in the kidney. The Malpighian corpuscles contained some erythrocytes and some proteinaceous material and fibrin. The proximal convoluted tubules also contained erythrocytes and some protein, while many of the endothelial cells were necrotic and contained pyknotic nuclei: an occasional macrophage was seen to be invading the necrotic areas (Figures 2.14 & 2.15). The distal convoluted tubules were often distended and contained proteinaceous material. Many endothelial cells were necrotic while others contained pyknotic nuclei and showed vacuolation of the cytoplasm. In the sheep that died from causes not associated with copper toxicity, the only changes noted were the presence of some protein and some erythrocytes in the Malpighian corpuscles.

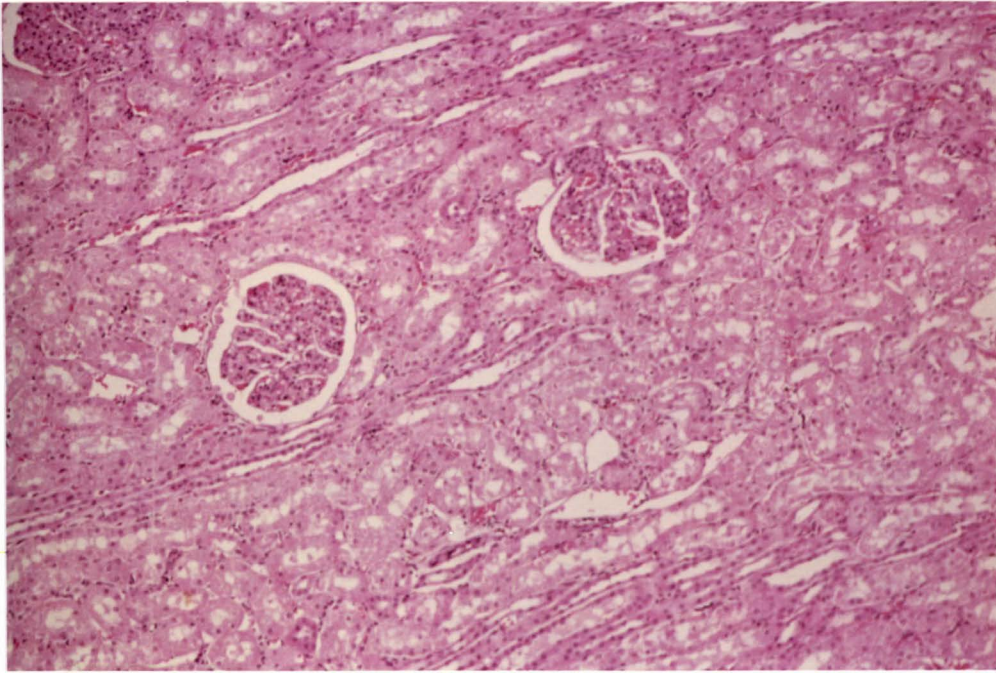


Figure 2.14: Histology of normal kidney tissue
(H. & E. stain) (100 X magnification)

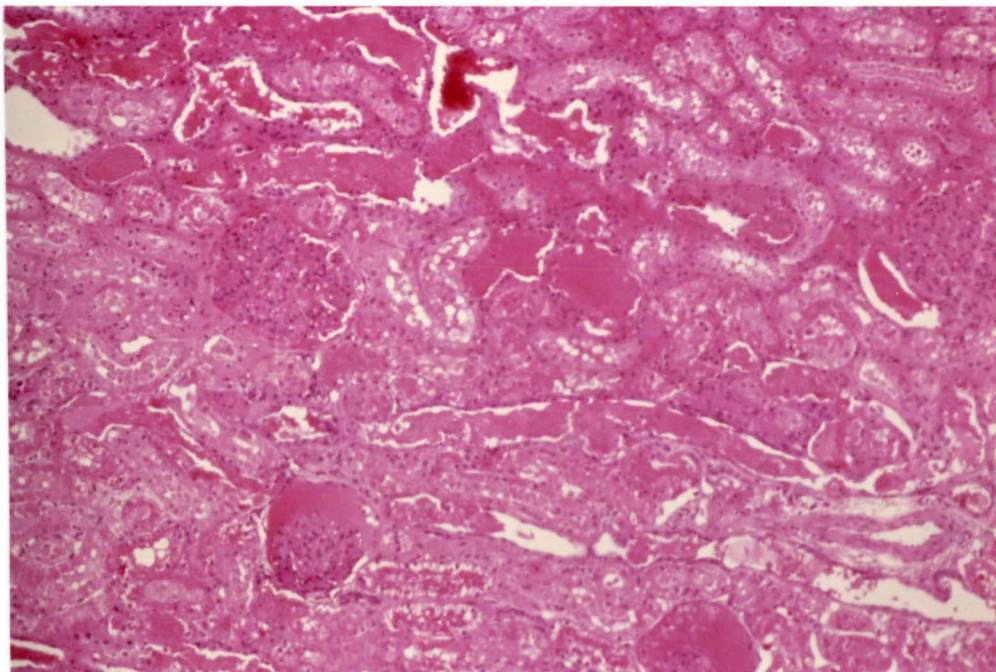


Figure 2.15: Histology of kidney tissue from a sheep that
died from copper toxicity (H. & E. stain) (100 X magnification)

Occasional greenish/black granules of copper were seen in the endothelial cells of the proximal convoluted tubules in the sections stained with rubeanic acid. The blue granules of haemosiderin and iron salts in the cytoplasm of the cells of the cortical labyrinth and in the Malpighian corpuscles were seen in the sections stained with Perl's iron stain. There were no blue granules present in the distal convoluted tubules.

Brain tissue

There were no significant histopathological changes in the three sheep that died from causes other than copper poisoning. The sheep that died as a result of copper toxicity did show changes that were interpreted as a degeneration. Some of the astrocytic nuclei were enlarged or distorted, or showed fragmentation and clumping of the chromatin. Fragmentation of the oligodendrocytic nuclei was seen in the white matter with some hydropic changes about the nuclei, indicating an early spongy change. The neurones showed signs of early degeneration with bizarre-shaped nuclei: these being enlarged, elongated, fragmented, T-shaped, or Y-shaped. These changes were more evident in the brain stem than in the cerebral cortex (Figures 2.16 & 2.17).

There was no evidence of the greenish-black granules of copper in any of the sections stained with rubeanic acid, and no significant changes were seen in the myelin in those sections stained with luxol fast blue.

Reaction at the injection site

In all sheep the reaction at the site of the injection was negligible. Of the 105 different injections given, on only twelve occasions was localised heat felt at the injection site at 24 hours, on twenty-four occasions a soft palpable reaction was palpated at 12-24 hours, and on two occasions a firm swelling was palpated at the

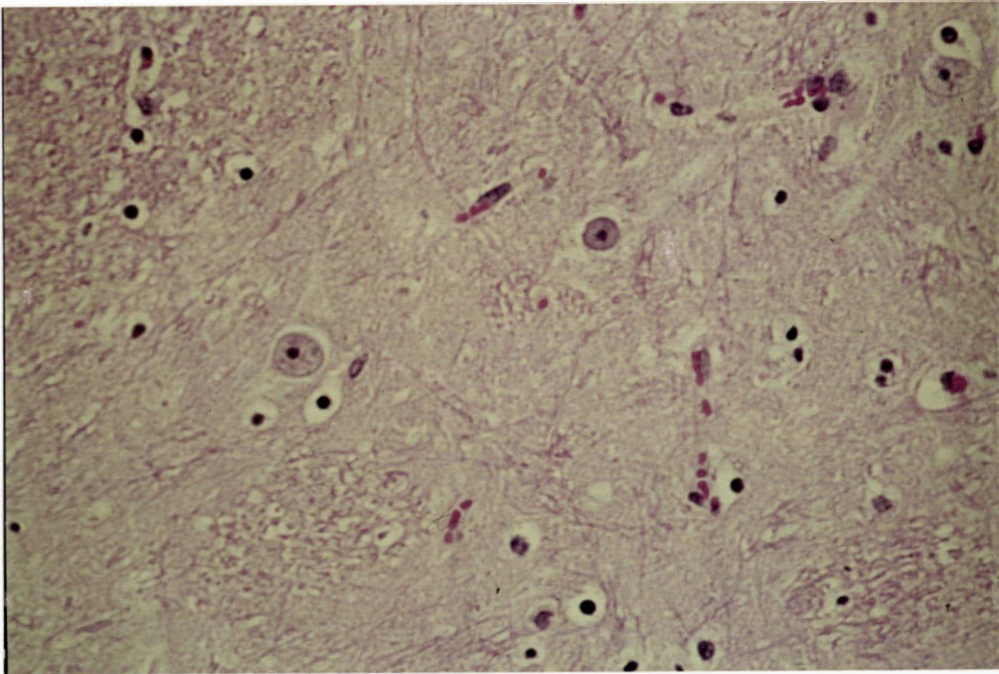


Figure 2.16: Histology of normal brain tissue
(H. & E. stain) (400 X magnification)

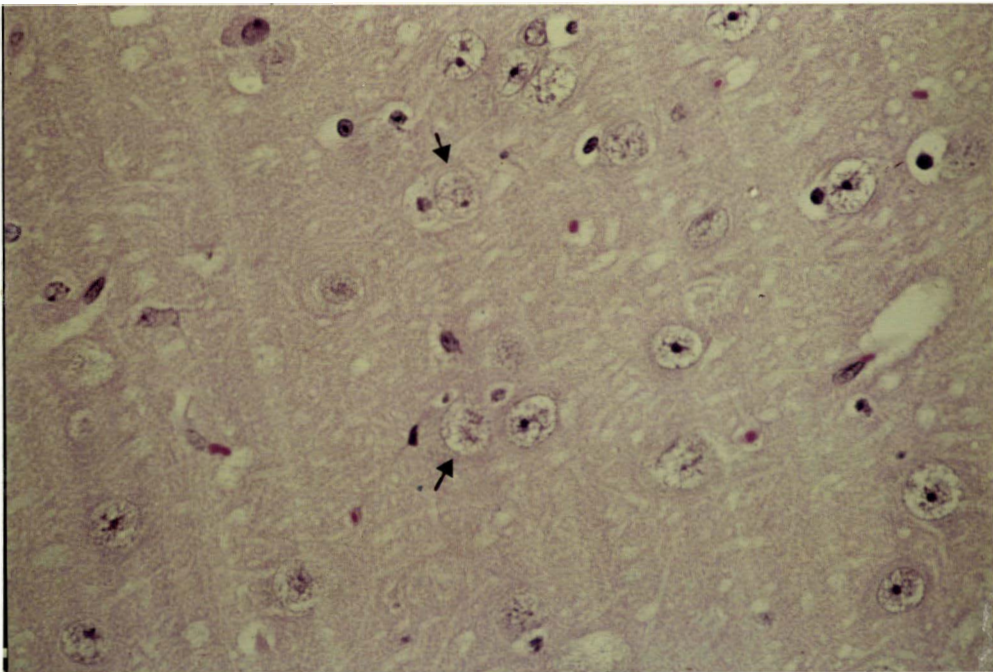


Figure 2.17: Histology of brain tissue from a sheep that
died from copper toxicity showing enlargement of astrocytic
nuclei (↑) (H. & E. stain) (400 X magnification)

injection site, but this diminished over 48 hours. Two sheep produced nodules, approximately 3 cm in diameter, which subsequently ulcerated four weeks after the injection. However there was no correlation between any reaction to the injection and liver copper content, or liver copper uptake, or liver enzyme response, or the total number of injections administered. Except for the two sheep in which ulcers occurred, the reaction at the sites of injection were minimal and no sheep showed any permanent disfigurement.

Bodyweight

Bodyweights were not significantly affected throughout the experiment, except in those sheep surviving the haemolytic crisis. These sheep lost 4-5 kg over the haemolytic period. This was mainly due to the inappetance which lasted for 3-4 days. The lost weight was regained over the following 4-5 weeks.

DISCUSSION

The initial study, using just two sheep, established the value of monitoring those biochemical parameters selected to determine the changes that occurred as the copper toxicity developed. These results warranted the expansion of the experiment to include a further nine sheep.

The blood parameters during the haemolytic crisis and just prior to death showed changes which were typical of a developing and terminal haemolytic anaemia. Unfortunately the haemolytic crisis was usually so sudden in onset that in some sheep the last blood sample obtained was taken before haemolysis became evident. As almost all iron in blood is contained in haemoglobin, the decrease in blood iron content parallels the decrease in blood haemoglobin content when haemolysis occurs.

The rise in PCV that showed in all sheep three to four days before the haemolytic crisis (Figure 2.2), was also observed by McCosker(1968), MacPherson & Hemingway(1969), and by Ishmael et al.(1972). These authors suggest that the increase in PCV may be due either to an increase in cell size or to dehydration. As there is no change in MCHC at this time, but there is an increase in plasma protein content, it would appear that the increase in PCV is most likely the result of dehydration.

The serial blood samples taken from sheep 237 during the haemolytic crisis indicated that up to 20% of erythrocytes lyse at the peak of the haemolytic crisis. However the intact erythrocytes still had a normal structure with an MCHC of between 31.9 and 36.0. The presence of Heinz bodies and the characteristic malformation of affected erythrocytes from sheep 237 confirms the changes at the time of the haemolytic crisis observed by Soli & Froslic (1977) and strongly suggests that this may result from two phenomena, lysis of erythrocytes, and splenic phagocytosis of crenated cells containing denatured haemoglobin.

As an MCHC greater than 40% is indicative of intravascular hemolysis (Schalm et al., 1975), the results indicate that in nine of the eleven sheep some haemolysis must have occurred on one to three occasions. This occurred 7 to 91 days before the appearance of clinical haematuria or death. The degree of intravascular haemolysis showed no relationship to the amount of copper injected, to the blood copper concentrations, or to the preexisting liver copper concentrations of the affected animals. Haematuria was not seen in those sheep undergoing a minor haemolysis as all the lysed haemoglobin is complexed to haptoglobins and this complex is removed by the reticulo-endothelial system (Schalm et al., 1975).

There was no correlation between blood copper concentrations and liver copper concentrations in this study. MacPherson et al., (1964), similarly found poor correlation between blood copper concentrations and liver copper concentrations, also when using sheep

of above normal copper status. Some studies (Suttle, 1976), have shown a correlation between blood copper content and liver copper content, but in most of these other studies the sheep used were of low or deficient liver copper status. It was at the time of the haemolytic crisis that there was a marked and variable increase in blood copper concentrations in different sheep. This could be attributed to the fact that the time of collection did not coincide with peak haemolysis. The highest blood copper level recorded was 8.92 ppm. The elevation in blood copper concentrations is comparable to the increases found by Marston (1952) and by more recent workers. McCosker (1968b) reported increases in blood copper levels 7 days before the haemolytic crisis and in some sheep 28 days before the haemolytic crisis. Similar findings were not seen in this experiment, but episodes of intravascular haemolysis did occur as was reported by McCosker. It would appear that the release of copper from the liver occurs very quickly and further it is very rapidly excreted in the urine which accounts for the high copper content in the kidney following death from copper poisoning.

The elevation of the activity of the liver enzymes SGOT and SDH in serum after each copper injection was not consistent. The amount of liver enzyme that appeared in serum showed no relationship to the amount of liver damage. It may depend either on the degree of liver necrosis or on an alteration in the permeability of the liver cell membrane. The latter change may be reversible but the important feature was the rise in serum enzyme activity that indicated liver damage (Kramer, 1980).

Sorbitol dehydrogenase is a liver specific enzyme and it has a short half-life (Kramer, 1980). It is a more sensitive measure of liver damage than SGOT, as following liver damage SDH activity is apparent before that of SGOT. The erratic pattern of enzyme response recorded in this study differed from the pattern seen by Todd & Thompson, (1963); MacPherson & Hemingway, (1969); and Ishmael et al., (1972). (Table 1.1). Their results showed a gradual change, with minor fluctuations, but a more rapid increase occurred closer to

the onset of the haemolytic crisis. However in their experiments the sheep received daily supplementation with copper, and were fed on diets with elevated copper levels, while in the current experiment the sheep received a weekly dose of copper. The use of SDH as an indicator of hepatic damage proved valuable in this experimental design, as the response to each dose could be measured. In addition, its short half-life ensures there is little risk of a carry-over effect that might persist until the next injection.

The most accurate picture of the progress of liver damage can be established from the histopathology of the affected liver. All the experimental sheep showed a gradual increase in the amount of cellular damage, and during the haemolytic crisis or at death the histological findings were consistent. This is in agreement with the findings of Ishmael et al. (1971b). The amount and size of copper granules indicated by the rubeanic acid stain increased with the increased liver cell damage. This finding is similar to the observations of Gooneratne et al. (1980). The extent of hepatic cellular necrosis varied with each sheep and was not apparently related to the dose of copper, the liver copper concentration, or to the release of SDH and SGOT.

The histological changes seen in the kidneys resembled those described by Ishmael et al. (1971b). The presence of granules of copper and of iron salts were seen in the proximal convoluted tubules as shown by Gopinath & Howell (1975). However in the sheep used in this study the copper granules were not as numerous or as dense as those described by Gopinath & Howell, nor as dense as those seen in the liver sections.

Status spongiosus as seen by Doherty et al., (1969), and by Morgan (1973) in the brain of some sheep dying from copper poisoning was not seen in the cases investigated in this study. However the astrocytic changes as described by Howell et al., (1974), were seen in all the sheep that died from copper poisoning. Suzuki & Kikkawa (1969) state that status spongiosus may be due to markedly swollen

astrocytes, to intramyelinic vacuoles, or to intra-axonal vacuoles and that these changes may not be differentiated with the light microscope. In the sheep studied in this experiment the changes in the central nervous system had been manifested as changes in the astrocytes and oligodendroglia.

Of the eight sheep that died following copper treatments, three died before the haemolytic crisis and showed only signs consistent with liver copper accumulation, but no lesions of copper toxicity. It is of interest that the two sheep that showed acute toxicity were those that had received the greatest number of copper injections (eleven). At autopsy these sheep showed signs typical of acute copper toxicity, with massive effusion of fluid into the pleural cavity, as described by Ishmael et al. (1971a).

Those sheep that died from copper poisoning had elevated copper concentrations in the liver and kidney, and similar pathological changes in the liver and in the kidney. The copper concentrations in the livers of sheep that died from copper poisoning showed a wide range of values immediately prior to death. These values ranged from 4488 ppm D.M. to 1404 ppm D.M. which suggested that the upper limit of measured copper in chronic copper poisoning can be both high and extremely variable. There was a very substantial loss of copper from the liver immediately preceding the haemolytic crisis. Those sheep that died from other causes also showed a significantly lower liver copper content at death than at the previous liver biopsy. It would appear that the liver of an unhealthy animal may lose copper at a high rate. Animals readily lose protein from the liver during periods of inappetance, (Mortimore, 1982), but no workers have alluded to a loss of copper. Further study may be needed to determine whether these losses are the result of the same mechanism. This apparent copper loss may be important when interpreting results of chemical analysis of liver samples taken from animals in low body condition or dead animals. It may give a false value of the true picture of a herd or flock in which the liver copper concentrations are being investigated. Such results may need to be interpreted with

caution. It is also possible that a similar loss from the liver may occur with other trace elements, e.g. selenium.

Kidney tissue copper levels were raised significantly in those animals which died from copper toxicity when compared to those which died from other causes. During the haemolytic crisis there was a release of a large amount of copper from the liver into the blood stream. This excess blood copper is rapidly filtered by the kidney (Goldfischer et al., 1970) with the result that copper concentrations in this organ become very high and that the kidney is probably the most useful tissue for confirmation of chronic copper toxicity.

The copper concentrations of brain tissue in the sheep that died from copper poisoning showed some elevation, in comparison to the copper levels of brain tissue obtained from animals which were clinically deficient, but concentrations from poisoned sheep were not significantly higher than those of normal sheep. These same findings were reported by Gooneratne & Howell (1979). Estimation of brain copper content would not be a useful aid in the diagnosis of copper poisoning.

The three sheep that survived the haemolytic crisis gradually returned to normal health. In these same sheep, the levels of both liver enzymes (SDH and SGOT) were elevated for up to three weeks after the last copper injection. The continued elevation of serum enzyme activity cannot be explained from the data available, particularly as the blood copper concentrations and the blood parameters had returned to normal. It may have been the result of continued hepatic cellular necrosis as no regeneration of hepatic cells was evident during this time. Further histopathological and histochemical study over this period of resolution may suggest a cause of these continuing high enzyme levels.

The average loss of liver copper in the sheep that survived the haemolytic crisis was an estimated 2.61 mg per day which indicated that there is probably some form of control of copper excretion.

Further it has been shown in this study that this excretory mechanism appears to eliminate more copper when the liver contains a high concentration of copper.

In these experiments it was noted that the mitotic activity of hepatic cells was observed only in those sheep that survived the haemolytic crisis. This mitotic activity was first observed in liver tissue collected two weeks after the haemolytic crisis. A similar observation was made by Manns (1978) while investigating the regeneration of ovine liver after the administration of a sublethal dose of sporidesmin. He found mitotic activity was not evident until 10 - 14 days after the injection of sporidesmin. This delay in regeneration is variable as sheep dosed with carbon tetrachloride showed regeneration of liver cells within two days of treatment (Manns, 1978). Bull et al., (1968), working with the pyrrolizidine alkaloid lasiocarpine, found that the antimitotic effect of the alkaloid persisted for at least three months. Therefore it would appear that the persistence of high levels of SDH and SGOT activity in the serum of the sheep that survived the haemolytic crisis may have been due to the delay in regeneration of the liver cells, and also may be due to the possible continuing necrosis of some cells after the peak of the haemolytic crisis. It could also be due to the failure of the cytoplasmic membrane as a result of continued hypoxia, to reestablish its integrity and maintain the cytoplasmic components within the cells.

Manns (1978), found only 9 - 12% of hepatic necrosis was necessary to induce mitosis and regeneration. As 50% necrosis was seen in the liver of those sheep that survived the haemolytic crisis, it would appear that high concentrations of hepatic copper and the continued administration of copper are capable of retarding this regenerative activity.

CONCLUSIONS

Copper injected into sheep in repeated doses in the form of copper calcium edetate is hepatotoxic. This may be assessed by the measurement of serum activity of SDH and SGOT which has escaped out of damaged hepatocytes, and by histological examination of liver tissue. The increase in enzyme activity is the earliest indicator of developing toxicity; SDH being the first enzyme to show elevated serum concentrations.

Immediately preceding the haemolytic crisis there may be a release of large amounts of copper into the circulation from approximately 50% of the hepatic cells. This release initiates a haemolytic crisis producing tissue hypoxia which results in death in some cases, or eventual recovery in others.

There is considerable individual variation between sheep in the amount of copper necessary to cause toxicity. This does not appear to be related to the total amount of copper injected or to the copper content of the liver tissue.

Diagnosis of copper toxicity can be based on clinical and post-mortem signs and on the liver copper content, but must be confirmed by liver histology and chemical analysis of kidney tissue.

CHAPTER 3: ASSESSMENT OF THE EFFECT OF VARIOUS STRESSORS ON THE
POTENTIAL FOR TOXICITY FROM COPPER THERAPY.

INTRODUCTION

In the field, veterinary remedies are administered to animals under a variety of conditions which may enhance or retard the translocation of the drug from its site of injection. These conditions include sex, age, breed, and liveweight, state of nutrition, hydration and pregnancy, and environmental temperature. There may also be the added effect of a disease, or the effect of a concurrent treatment of the animal with other chemicals. Therefore in evaluating a new therapeutic agent for sheep, for example, copper calcium edetate, it is important to test the product under various circumstances which are similar to those encountered on New Zealand sheep farms.

Lewis et al., (1981), reported on the toxicity of copper calcium edetate for lambs and showed that the therapeutic dose and the toxic dose in lambs is almost identical. A copper calcium edetate dose rate of 3 mg Cu/kg bodyweight has caused deaths in adult sheep (Mahmoud & Ford, 1982): the incidence being 1 in 2,000 (Ishmael et al., 1969). After administering a dose of 50 mg of copper (approximately 1 mg Cu/kg bodyweight) deaths have occurred in some sheep while being transported (Skinner, 1960) or handled (Ross, 1964).

In this section of the thesis experiments are described which were designed to evaluate the effects of certain stressors when superimposed on a conventional copper dosage regime which was known to cause only minor changes in relevant parameters (Chapter 2). Having established that the levels of activity in the serum of the liver enzymes SDH and SGOT are the earliest indicators of hepatic damage caused by copper, the measurement of these was used in each animal to evaluate the toxicity of copper calcium edetate under each respective condition of stress. The stressors chosen were those

thought to be common on many New Zealand sheep farms, and typical of the circumstances when copper may be given about the same time. Accordingly experimental conditions were established which were similar to partial starvation, dehydration, excessive heat or cold, and parasite infection. Concurrent treatment with either an anthelmintic or a parasiticide was used and also the effect of copper on pregnant ewes was measured.

In a typical New Zealand sheep flock the age of the ewes ranges from two years old to five or six years old. The bodyweight of these may range from 35 kg to 65 kg, and there may even be more than one breed of sheep present. The dose rate selected for most of these experiments was 3 mg Cu/kg bodyweight, whereas the dose recommended by the product manufacturer is approximately 1 mg Cu/kg bodyweight, giving a total dose of 50 mg of copper to an adult ewe. The premise for this dose rate of 3 mg Cu/kg bodyweight was that a light (35 kg) ewe may inadvertently be injected twice, an accident that can readily occur when large numbers of animals are being treated. Should this occur, then the same 35 kg ewe would receive a total dose of 100 mg of copper equivalent to a dose rate of approximately 3 mg Cu/kg bodyweight.

MATERIALS AND METHODS

Treatment with copper

Fourteen groups of sheep were subjected to their respective stressors as summarised in Table 3.1. The sheep used were all aged Romney ewes, except for the parasite experiment in which younger animals were considered more typical of the true field situation. The treated animals were injected subcutaneously with copper calcium edetate¹, at a dose rate of either 2 mg Cu/kg bodyweight or 3 mg Cu/kg bodyweight. The control animals were not treated with copper, except in the parasite experiment in which the control animals were "worm-free" but did receive copper supplementation. The general arrangement for these experiments is set out in Table 3.1.

¹ Coprin Multidose, Glaxo (N.Z.) Ltd.

Stressor	Cu dose mg/kg	No. treated	No. control	Feed	Water	Special treatment
Triple dose	3	5	0	pasture	*	nil
3 x 1	3	5	0	pasture	*	nil
Starve-triple	3	4	0	nil	nil	starved 48 hours
Starve-3 x 1	3	4	0	nil	nil	starved 48 hours
Dehydration	3	4	2	hay	nil	750mls blood removed
X Cold	2	4	4	hay	*	shorn/room temp. 6°C
✓ Cold	3	4	0	hay	*	shorn/room temp. 6°C
✓ Heat	2	4	4	hay	*	room temp. 41°C
✓ Heat	3	3	0	hay	*	room temp. 41°C
Pregnancy	3	3	0	pasture	*	4 months pregnant
Insecticide	3	4	4	pasture	*	diazinon
Anthelmintic	3	4	2	pasture	*	oxfendazole
Parasitism	2	4	2	pasture	*	see text
Parasitism	3	4	4	pasture	*	see text

Table 3.1: Details of housing, management, stressors, and copper administration, and schedules to all groups of sheep.

X Temperature

Stressors

The initial groups of sheep were not subjected to any stressor but received either a single dose of copper at 3 mg Cu/kg bodyweight (triple dose), or three separate doses of 1 mg Cu/kg bodyweight given at hourly intervals (3 x 1) (Table 3.1). The cold-stressed animals were shorn, placed in crates in the cold room at a temperature varying between 5.0 and 6.5°C, and rectal temperatures were recorded daily. Those sheep subjected to the hot environment were also placed in crates and had rectal temperatures and respiratory rates recorded twice daily.

In this experiment "dehydration" was achieved by bleeding. Each sheep had 750 ml of blood removed (approximately 25% of total blood volume) immediately before the treatment with copper. In these dehydrated sheep the plasma protein decreased by 0.52 gm/100ml (7%) at 24 hours and then returned to normal.

Liver biopsy samples were not collected from the group of pregnant ewes because of their advanced pregnancy.

In the experiment involving parasitised animals, nine-month old Romney hoggets were used. The "worm-free" control animals were selected from animals with a zero faecal egg count, and then each sheep was treated once weekly for six weeks with 180 mg oxfendazole². The "parasite-infected" animals had faecal egg counts in excess of 1570 e.p.g. Each hogget that died, and the two parasitised hoggets that received 3 mg Cu/kg bodyweight and survived, had their gastrointestinal contents submitted for total worm counts. The surviving lambs used for total worm counts were killed after the second liver biopsy had been taken. It had previously been determined that benzimidazole anthelmintics had no effect on the biochemical parameters measured in this experiment.

²Synanthic, Syntex (N.Z.) Ltd.

Those sheep treated with anthelmintic received an oral dose of 30 ml of oxfendazole containing 22.65 gm of active ingredient per litre. The sheep treated with insecticide were immersed for 30 seconds in a bath containing the organophosphate, diazinon, at a concentration of 300 mg per litre.

Collection of blood samples

Blood and serum samples were taken from each animal immediately before treatment and at 48 hours and 96 hours after the copper was administered. The blood samples were analysed for haemoglobin, packed cell volume, plasma protein and icteric index, and the MCHC was calculated. Blood was also digested in hydrogen peroxide and nitric acid and analysed for the copper content. Serum samples were analysed for SDH and SGOT activity as described in Chapter 2.

Liver biopsies, for chemical analysis of the copper content, were taken three days before the time of the copper injection and again four days after the copper injection. The bodyweight of each sheep was recorded at the same time.

Any sheep that died during the period of experimentation was subjected to a post-mortem examination. Tissue samples of liver, kidney and brain were placed in 10% buffered formol saline for histological examination, and further samples of the same tissues were frozen awaiting analysis for their copper content (Appendix 1).

RESULTS

In those ewes subjected to cold or dehydration, those which were pregnant, or those which had been treated concurrently with insecticide or anthelmintic, no deaths occurred and no significant findings were recorded. In the groups subjected to the stressors of heat, starvation or parasitism, the results were much more dramatic. Nine of these 23 sheep died.

The results showing the number of animals that exhibited intravascular haemolysis, the changes in liver enzyme activity and the number of deaths are given in Table 3.2. These results are further summarised in Table 3.3 into groups of sheep in which deaths did occur and those in which no deaths occurred.

There were no significant differences in bodyweight change for any sheep throughout its experiment. Assuming that liver is 1.13% of bodyweight and that it is 29.4% dry matter (van Ryssen, 1980), then the calculated mean liver retention of the parenteral copper was 65%. There was no significant variation in retention rate between any of the groups. Blood copper increased by a mean of 0.5 ppm at 48 hours for all experiments.

Of the sheep in the initial group that received 3 mg Cu/kg bodyweight, one died of copper toxicity. There were no significant differences between those sheep receiving a triple dose of copper, and those sheep receiving the same dose but divided into three equal doses and given at hourly intervals.

The rectal temperatures of the sheep in the cold environment were reduced by 0.6°C after 36 hours and they remained at this level throughout the experiment. The food intake of these sheep was high at approximately 1.6 kg D.M./day. The liver enzyme concentrations of these sheep did not peak until 96 hours after treatment; a delay that also occurred with the rise in copper concentrations of the blood.

In those sheep exposed to the hot environment, rectal temperatures were raised by 0.6°C after four hours, and remained at this elevated level while the sheep remained in this hot environment. The respiratory rates increased from a mean of 98/minute to 172/minute in the first four hours, and then returned to a steady rate of 130/minute. These sheep drank large volumes of water, but food intake was severely reduced to approximately 0.2 kg D.M./day. Two of the four sheep in the group receiving 3 mg Cu/kg bodyweight

Stress	Cu dose mg/kg	No. of Animals	No. show Haemol.	S. D. H.			S. G. O. T.			No. died
				norm	mid	high	norm	mid	high	
triple dose	3	5	0	2	2	1	1	2	2	1
3 x 1	3	5	1	3	1	1	1	3	1	0
starve	3	4	1	1	1	2	0	0	4	2
3x1starve	3	4	2	1	1	2	0	1	3	2
dehydr	3	4	0	2	0	2	2	0	2	0
heat	41°C	2	4	0	4	0	0	4	0	0
heat	41°C	3	3	1	0	2	0	2	0	1
cold	6°C	2	4	0	0	2	2	0	4	0
cold	6°C	3	4	0	2	1	1	0	3	1
preg	3	3	0	2	1	0	3	0	0	0
insect	3	4	0	2	1	1	0	1	3	0
anthel	3	4	0	1	2	1	2	0	2	0
parasit	2	4	0	1	0	3	1	0	3	2
parasit	3	4	0	2	0	2	2	0	2	2

S.D.H. levels

norm = < 20 i.u./ml

mid = 21 - 200 i.u./ml

high = 201 + i.u./ml

S.G.O.T. levels

norm = < 75 i.u./ml

mid = 76 - 400 i.u./ml

high = 401 + i.u./ml

Table 3.2: Results of haemolysis, liver enzyme increases, and number of deaths in animals from all groups.

Effect of stressors	Deaths per group	No.with haemol.	S D H			S G O T		
			norm	mid	high	norm	mid	high
No deaths	0/23	0	9	7	7	7	8	8
Some deaths	9/23	4	9	4	9	3	7	12
			(a)			(b)		

Note: one sheep died before serum was collected.

(a) and (b): see text.

Table 3.3: Summary of results of Table 3.2 combining stressor groups exhibiting deaths and stressor groups in which no deaths occurred.

died before treatment started, and the other 36 hours after copper administration. Neither sheep showed evidence of copper toxicity either in histological changes of tissues taken after death, or from chemical analysis. Hyperaemia of the intestinal tract and enlargement of the gall bladder were seen in both sheep. These findings were suggestive of salmonellosis but this was not confirmed bacteriologically.

Fifty percent (four out of eight) of the sheep that were starved died from copper toxicity. These four sheep showed an increase in plasma protein of 1.125 gm/100 ml (14%), in comparison with a 0.275 gm/100 ml (4%) increase in those sheep that survived. Plasma albumin levels did not increase in the surviving sheep, but could not be measured in those sheep that subsequently died, because of the extensive intravascular haemolysis. Blood copper concentrations at 48 hours had increased by 1.84 ppm Cu in those sheep that died, in comparison with 1.32 ppm Cu in those sheep that survived.

Sheep no.	"Worm-free"				"Infected"			
	120	123	124	128	104	105	122	126
Bodyweight: kg.	20.5	20.9	25.0	20.9	25.5	23.2	19.1	20.0
Serum pepsinogen	0.2	0	2.0	0	0.4	0	0.2	0.8
Faecal egg count	0	0	0	0	3,650	2,400	7,050	4,600
Fate	sl.	sl.	died	died	died	died	sl.	sl.
Haemonchus	0	0	0	0	100	600	400	100
Ostertagia	0	0	0	0	100	700	700	1,100
Trichostrongylus	0	0	0	0	1,000	800	300	800
Cooperia	0	0	0	0	0	400	1,000	100
Trichostrongylus	0	0	0	0	600	400	1,300	200
Nematodirus	0	0	0	0	300	800	0	0
Chabertia	0	0	0	0	20	20	30	20
Oesophagostomum	0	0	0	0	60	20	210	20
Trichuris	0	0	0	0	0	0	30	0
Moniezia	0	0	0	0	pres	pres	present	0

sl. = slaughtered

Table 3.4: Results of bodyweight, serum pepsinogen levels, faecal egg counts and total worm burdens of hoggets given copper calcium edetate at a dose rate of 3 mg Cu/kg bodyweight.

Sheep no.	"Worm-free"		"Infected"			
	103	106	107	108	109	110
Bodyweight: kg.	27.3	23.6	30.0	17.3	24.1	24.5
Faecal egg count	0	0	1,570	1,600	2,100	2,750
Fate	survived		died	survived		died
Ostertagia			300			200
Trichostrongylus			2,000			5,900
Cooperia			1,100			300
Trichostrongylus			1,600			1,100
Nematodirus			100			1,700
Oesophagostomum			0			10
Chabertia			20			0
Trichuris			0			20
Moniezia			present		present	

Table 3.5: Results of bodyweight, faecal egg count and total worm counts of hoggets given copper calcium edetate at a dose rate of 2 mg Cu/kg bodyweight.

Sheep no.	Stressor	Copper dose mg/kg	Liver Cu ppm	Liver score H&E	Kidney Cu ppm D.M.	Kidney score H&E
11.05	triple	3	661	5	134	3
13.75	starve	3	833	4	41	4
13.155	starve	3	1129	4	66	4
14.496	starve	3	735	4	54	4
14.1086	starve	3	667	4	143	5
19.61	heat	3	235	2	21	4
26.107	parasite	2	123	4	123	4
26.110	parasite	2	752	4	168	3
24.104	parasite	3	587	3	52	3
24.105	parasite	3	801	3	92	3
24.124	"worm-free"	3	*	5	*	4
24.128	"worm-free"	3	747	4	82	3

* no tissue analysed

Histology key:

Liver H & E

1. No significant findings
2. Occasional macrophages & regular nuclei
3. Necrotic cells present
4. Pyknosis & necrosis, & aggregations of macrophages
5. 50% of nuclei enlarged, vacuolation present & aggregations of macrophages

Kidney H & E

1. Some protein in proximal tubules
2. Some necrosis & vacuolation in cells of proximal tubules
3. Pyknosis & necrosis in proximal tubules & macrophages present
4. Pyknosis and necrosis of tubules & macrophages infiltrating
5. Most tubules necrotic or distended, some pyknosis and invading macrophages

Table 3.6: Results from post-mortem material of sheep that died.

The hoggets in the parasite trial were the most adversely affected of any groups of sheep, following the copper supplementation. Two of the infected and two of the "worm-free" hoggets, which received 3 mg Cu/kg bodyweight, died 18-24 hours after treatment. Copper toxicity was confirmed as the cause of death. The two surviving infected sheep had an increased blood copper concentration of 0.9 ppm Cu at 48 hours, in comparison with the two surviving "worm-free" hoggets which had an increase of 0.4 ppm Cu. Table 3.4 gives the bodyweight, faecal egg count and serum pepsinogen levels at the time of injection, and the total worm burdens at the time of death or at slaughter.

Two of the infected sheep that received 2 mg Cu/kg bodyweight died at 45 and 72 hours respectively. The deaths were confirmed as being the result from copper toxicity. The two surviving infected hoggets and the two "worm-free" hoggets had blood copper concentration increases of 0.7 ppm Cu at 48 hours, whereas the infected hogget that died at 72 hours had an increase in blood copper concentration of 1.7 ppm Cu at 48 hours.

The three infected hoggets which were still alive at 48 hours had an icteric index of 50-75 units as compared with the non-infected hoggets which had 5 and 10 units respectively. MCHC levels did not indicate intravascular haemolysis. Table 3.5 gives the bodyweights, faecal egg counts and total worm burdens of these six hoggets.

Deaths:

Post-mortem examination, histopathology and chemical analysis of tissues confirmed that the deaths were the result of copper toxicity. Table 3.6 summarizes these findings.

DISCUSSION

It would appear from the results of these experiments, that the dose rate of copper used (3 mg Cu/kg bodyweight) is close to a toxic dose. One sheep which received this dose rate, but which was deliberately not subjected to any additional stress, died as a result of copper poisoning. Mahmoud & Ford (1982) have reported losses in the field in adult ewes that also had each received a dose of 3 mg Cu/kg bodyweight as copper calcium edetate. Therefore it is suggested that any stressor that will enhance the toxic potential of copper calcium edetate administered at this dose rate may be identified by measuring the changes in certain biochemical parameters in sheep subjected to that stressor and treated with copper calcium edetate. The responses may be death, or high increases in the biochemical parameters used to assess hepatotoxic damage.

In these experiments losses occurred in those groups subjected to heat, starvation, and to gastrointestinal parasites. In the other stressed groups there were no deaths as a result of the copper supplementation. In fact, the increases in the biochemical parameters that indicate hepatotoxicity were significantly lower in these sheep, than in the surviving sheep from the groups in which deaths occurred. There was no apparent difference in the biochemical parameters measured, between those sheep receiving 3 mg Cu/kg bodyweight as a single dose, and those receiving it in three doses of 1 mg Cu/kg bodyweight, each an hour apart.

Forty-eight hours starvation prior to the copper injection resulted in death in four out of the eight sheep treated. In published accounts of deaths following parenteral copper administration, it is apparent that in some cases the animals have been subjected to stressors, and especially to the stress of transport (Skinner, 1960) or handling (Ross, 1964). It would appear likely that sheep subjected to these stressors have been deprived of food and water for 24 to 48 hours before treatment: therefore the precipitating cause of toxicity is most likely to be starvation. The

sheep subjected to heat also showed an adverse response to the parenteral copper. These sheep were in a state of partial starvation as their feed intake was low.

In the sheep which died there was a marked increase in plasma protein, and in particular in the plasma albumin fraction. This factor is of special interest as albumin is the carrier of copper in the blood, from the injection site to the liver. The enhancement of toxicity may be the result of increased amounts of copper being presented to the liver because of the increased plasma albumin.

When the effects of copper supplementation were measured in sheep affected with gastrointestinal parasitism, several points arose. In the group receiving 3 mg Cu/kg bodyweight, half of the animals died of copper toxicity but the deaths occurred in both the infected and the worm-free animals. The predisposing cause was probably one of age as these animals were only nine months old. Lewis et al., (1981), reported that young sheep are more susceptible to copper toxicity. It seems likely that this is the result of younger animals absorbing drugs more rapidly from subcutaneous sites than older animals (Ballard, 1978). Ballard suggested that the difference is due to an increase in the thickness of subcutaneous tissue with age, as well as to changes in the composition of subcutaneous fat tissues.

However when the dosage of copper was only 2 mg Cu/kg bodyweight, losses from copper toxicity still occurred, but only in those animals affected with a heavy burden of gastrointestinal parasites. The blood copper content of these sheep that died increased by 1.7 ppm in comparison with 0.7 ppm in those that survived. The parasite burden carried in the infected sheep (Tables 3.3 & 3.4) would not be uncommon under some New Zealand farming situations. In these experiments the faecal egg count was much higher than that expected from animals carrying this total worm burden. This is probably due to a large increase in faecal egg concentration as a result of the clinically affected sheep losing

their appetite prior to death or slaughter. It is also possible that the sheep treated with copper may have lost some of their worm population after the copper treatment (Charleston, pers. comm.).

High parasite burdens can result in a large loss of protein from the intestine (Symons et al., 1974). This loss may produce modified protein metabolism leading to increased plasma albumin, or the induced inappetance caused by the parasite burden (Sykes & Coop, 1976; Sykes & Coop, 1977), may influence protein metabolism, to produce the same effect. As suggested earlier, this increased plasma albumin may enhance copper toxicity by increasing the uptake of copper from the injection site and presenting copper to the liver at a higher concentration.

In seven of the nine sheep that died from copper poisoning in these experiments, the serum levels of SDH activity and of SGOT activity did not rise significantly. In each of these cases intravascular haemolysis had occurred (Table 3.3 (a) and (b)) and the technique for measuring SDH activity specifies that the serum must be free of haemolysis, but the same condition is not specified for the SGOT technique. Presumably in both cases the free haemoglobin in the haemolysed serum utilized the enzyme substrate provided for the test so that little enzymic reaction was able to occur. In those sheep in which the MCHC value did not rise above 40%, it is possible that any free haemoglobin in the serum was bound to the serum haptoglobin, and therefore was not available to interfere with the reaction.

CONCLUSION

When copper calcium edetate is injected into sheep its toxicity may be potentiated if the animal is subjected to certain management stressors. These include 48 hours deprivation of food and water, exposure in an environment in excess of 40°C, and the presence of gastrointestinal nematodes. Young sheep are more susceptible to the toxic effects of copper calcium edetate than are adult sheep.

CHAPTER 4: ABSORPTION RATES OF DIFFERENT COPPER FORMULATIONS
ADMINISTERED UNDER VARIOUS CONDITIONS

INTRODUCTION

When all other variables are controlled, the differences in toxicity between the various parenteral copper preparations is dependent on the rate of translocation of copper from the injection site (Suttle, 1981b). The translocation rate of a drug is affected by the volume injected, the solids to vehicle ratio, and the particle size of the drug in suspension. It is also affected by the presence or absence of pharmaceutical agents such as suspending agents and by the pH, the tonicity of the formulation, the surface area of the depot formed at the injection site, the physicochemical properties of the drug, and the nature of the vehicle (MacDiarmid, 1983).

In 1977 a formulation of copper calcium edetate at a concentration of 25 mg Cu/ml, in a multidose container¹, was released for the correction of copper deficiency in cattle, in New Zealand. Unfortunately some 35 deaths in the 600,000 cattle injected were recorded, not as a result of copper toxicity, but from an anaphylactoid reaction. The agent that appeared responsible for this reaction was polyvinyl pyrrolidone (PVP), (Easingwood, 1981). Polyvinyl pyrrolidone was included as a suspending agent and also used to delay the absorption of copper from the injection site. Because of the ability of this product to translocate copper from the site of injection to ruminant liver without causing undue reaction at the injection site, the manufacturers reformulated the product by excluding the PVP and they doubled the concentration of the copper calcium edetate. The translocation rate of this new formulation², (hereafter referred to as the high concentration formula), had to be assessed, and its safety for use in sheep evaluated.

When evaluating the toxicity of copper calcium edetate in sheep which had been subjected to various stressors (Chapter 3), it was apparent that heat and starvation enhanced toxicity. It was decided

¹ Coprin Multidose, Glaxo (N.Z.) Ltd.

² Coprin, Glaxo (N.Z.) Ltd.

* In this chapter absorption refers to the movement of copper from the site of administration to the blood circulation.

to determine whether this enhanced toxicity was the result of an increased translocation rate, or due to some other factors. Differences in formulation may alter the translocation rate of copper (MacDiarmid, 1983), but other factors have not been described.

MATERIALS AND METHODS

In this series of experiments several proprietary copper preparations were administered. The low concentration formulation of copper calcium edetate contained 25mg of elemental copper per millilitre, and the high concentration formulation of copper calcium edetate contained 50mg of elemental copper per millilitre.

Stressors

The two sheep used in the pilot experiment described in Chapter 3, were injected subcutaneously with 50 mg of copper as copper calcium edetate in the low concentration formulation, (approximately 1 mg/kg bodyweight), 48 hours after entering the hot room which was maintained at a temperature of 40.0 - 41.5°C. Blood samples were collected immediately before treatment, and then at hourly intervals for 9 hours. The blood parameters of PCV, haemoglobin, plasma protein were measured for each sample, and the MCHC calculated. Blood samples were also digested and analysed for copper content. Blood uptake rates of copper were expressed as changes in the blood copper concentration over time, using the regression line from the start of sampling to the peak of blood copper concentration.

As described in Chapter 3, starvation and deprivation of water for 48 hours were the stressors that appeared to make sheep most susceptible to copper toxicity. A pilot experiment was carried out in which two sheep were deprived of food and water for 48 hours. These sheep were then injected subcutaneously with copper calcium edetate in the low concentration formulation at a dose rate of 2 mg Cu/kg bodyweight. Blood samples were collected immediately prior to

the copper injection and then hourly for 12 hours. Each blood sample was analysed for copper content. Regression lines were fitted to the raw data to assess the increase in blood copper over time.

The result achieved in the pilot experiment warranted an expanded trial. This was designed to confirm the early result, and to determine the duration of starvation required before blood copper uptake rates were significantly affected. Twenty-eight adult sheep were randomly placed into six groups and subjected to varying periods of starvation as set out in Table 4.1.

No. in group	Duration of starvation	Access to water
5	72 hours	no
4	72 hours	yes
5	48 hours	no
5	24 hours	no
5	12 hours	no
4	Control	yes

Table 4.1: Schedule of food and water deprivation of sheep used when determining copper uptake rate.

A liver biopsy was taken from each sheep six days before the copper supplementation, and again at the end of the experiment seven days later. The sheep had access to adequate pasture from the time of the first liver biopsy until the commencement of the starvation period. Only the control group had access to feed during the period of blood collection. Each sheep was injected subcutaneously with copper calcium edetate in the low concentration formulation at a dose rate of 2 mg Cu/kg bodyweight. Blood samples were collected immediately prior to the copper injections and then at hourly

intervals for 12 hours. Blood samples were also collected at the start of the respective period of starvation. Bodyweights of each sheep were recorded at the start of the period of starvation, and at the completion of the experiment.

The blood samples collected at the commencement of the starvation period and at the time of copper injection were used in an attempt to quantify the effects of starvation. Packed cell volume of the blood was measured. Plasma was assessed for glucose content using glucose oxidase³, and for the presence of ketones using the nitroprusside method⁴. Total plasma protein was determined using a refractometer, and plasma albumin using the bromocresol green reaction.

The bloods were also digested and analysed for copper content. Regression lines were fitted to the copper concentration data from the pre-injection time to the peak blood copper levels to assess the increase in blood copper concentration over time.

Copper formulations

The first experiment was designed to evaluate the increase in blood copper concentration, and the total uptake of copper from two oral copper preparations, namely, a "Mineral Premix" containing copper edetate⁵, (a copper chelate), and copper oxide needles⁶.

Two groups of four, aged, Romney ewes were selected for this experiment. Liver biopsies were obtained on days 0 and 5 of the experiment. The sheep were penned and maintained on a diet of hay containing 3.6 ppm D.M. of copper. The mineral premix-treated group received an oral dose containing 0.33 mg Cu/kg bodyweight - the manufacturer's recommended dose; and the copper oxide group were dosed with 2.86 gm of copper contained in 7 gm of copper oxide needles, in a gelatine capsule.

³ Mercktest 3393, E. Merck, Germany

⁴ Acetest tablets, Ames Division, Miles Laboratories, Australia

⁵ Mineral Premix, May & Baker (N.Z.) Ltd.

⁶ Copper oxide, Merck Ltd.

Blood samples were collected immediately prior to copper supplementation and then hourly for 12 hours. The sheep in the copper oxide group had liver biopsies taken every fourteen days for 84 days.

Blood parameters measured on the collected samples were PCV, haemoglobin content and total plasma protein. Each blood sample was also digested and analysed for copper content. Regression lines were fitted to the data from pre-injection time to peak blood copper levels to assess the increase in blood copper concentration over time. Liver biopsy samples were analysed for copper content.

To assess the uptake rate of the high concentration formulation as described in the introduction, twenty aged Romney ewes which weighed approximately 50 kg were used. The numbers of sheep used and the dosage of copper used are set out in Table 4.2.

Formulation	No.of sheep	Copper dosage	Dose volume
Low conc.	4	50 mg	2 ml
High conc.	5	50 mg	1 ml
High conc.	6	100 mg	2 ml
High + water	5	50 mg	2 ml

Table 4.2: Design of an experiment to compare translocation rates of different injectable copper formulations.

Blood samples were collected at the start of copper supplementation, hourly for 16 hours, and then at 24, 36, and 48 hours. These blood samples were digested and analysed for copper concentration. Regression lines were fitted to measure the increase in blood copper concentration.

Five of the sheep receiving 100 mg of copper contained in the high concentration formulation were also used to assess the toxicity of this new formulation. These sheep had a liver biopsy taken before the copper supplementation and again 7 days after treatment. The liver tissue was analysed for copper content. Serum from blood samples taken from these sheep at 0, 48, and 96 hours, was assessed for SDH and SGOT activity.

These sheep were penned throughout the experiment and had access to water and meadow hay.

The statistical analysis applied to the recorded data was an analysis of variance one-way command. The F-ratio was used as the test statistic.

RESULTS

Heat

The blood parameters of plasma protein, PCV and haemoglobin varied within 5% of the mean throughout the 9 hour bleeding period, while the MCHC remained almost constant.

The regression line of increase in blood copper concentration over time had for the two sheep slopes of 0.0387 and 0.0211 mg Cu/litre of blood per hour respectively, with a mean slope of 0.0299 mg Cu/litre/hour.

Starvation

The regression line describing copper uptake for the two pilot sheep had a slope of 0.0897 and 0.0786 mg Cu/litre of blood per hour respectively, with a mean of 0.0842 mg Cu/litre/hour.

Table 4.3 gives the results of the blood parameters measured, and the changes in bodyweight. The regression lines of copper uptake are given in Table 4.6. The liver biopsies taken before and after treatment showed that the liver stored 56% of the administered copper.

Parameter	Starvation period in hours					
	72	72+H2O	48	24	12	0
Kg loss	6.3	5.1	5.0	5.6	3.7	0
PCV change	+0.10	+0.10	+0.04	+0.06	+0.10	0
Plas prot change $\frac{gms}{100ml}$	+0.7	+0.9	+0.5	-0.3	+0.3	0
Glucose change $\frac{gms}{100ml}$	-2.3	-2.5	-1.5	-1.4	-1.0	0
Albumin change $\frac{gms}{100ml}$	+0.96	+0.75	+0.48	-0.10	-0.14	0
Alb. % change	+10.2	+7.0	+4.5	-0.3	+2.2	0

Table 4.3: Mean blood parameter changes of sheep undergoing various periods of starvation immediately prior to copper supplementation.

Premix

The blood parameters measured showed no significant variation throughout the 24 hours of uptake. Liver biopsies showed a mean increase of 52 ppm of copper. Assuming a total liver weight of 1.13% of bodyweight and a 29.4% dry matter content (van Ryssen, 1980), the liver retention of the copper was 46% of the administered dose. The hay diet of these sheep contained 3.7 ppm copper, < 0.8 ppm molybdenum, 0.16% sulphur, and 60 ppm of iron.

Blood copper concentration peaked at a mean of 7 hours (+1 hour) after copper administration, and the increase in blood copper concentration had a mean regression line of 0.0210 mg Cu/litre/hour.

The serum enzyme levels of SDH and of SGOT remained within the normal limits in every sheep.

Copper oxide needles

The blood parameters of PCV, haemoglobin and plasma protein did not vary significantly throughout the experiment. Blood copper levels remained almost constant during the 12 hours of sampling and showed a mean regression line of -0.0093 mg Cu/litre/hour.

The liver biopsies taken throughout the experiment gave liver copper concentrations as seen in Table 4.4.

Sheep no.	Liver copper content: ppm DM			
	7	163	237	274
Day 0	443	562	1203	353
14	1016	507	979	178
28	1106	1284	1275	3261
42	1284	1410	1184	1484 *
56	1422	1280	1060	
70	1423	1106	1140	
84	1306	1104	1081	

* = death

Table 4.4: Liver copper content of sheep after administration of copper oxide needles.

Sheep 274 failed to recover from the anaesthetic given 6 weeks after the treatment with copper oxide needles. On examination of the liver, lesions typical of facial eczema were seen. The abomasal washings were recovered, and analysed for copper content. The 15.77 gm of abomasal washings contained 2854 ppm of copper, indicating that

6.73 gm of the 7 gm of the copper oxide needles had been absorbed or lost over the 6 week period. The estimated amount of copper retained in the liver was 4.9% of the administered dose, after 6 weeks. Only two of the four sheep were considered in these calculations as sheep no. 274 had liver lesions of facial eczema, and sheep no. 237 had erratic liver copper levels on analysis of biopsy samples, possibly also associated with facial eczema changes.

Copper calcium edetate

The uptake rate of copper measured in blood samples showed a variation when different formulations of copper calcium edetate were used. There was a non-significant difference between low and high dose rates, but a significant difference occurred when the formulation was diluted with water, ($P > 0.01$), or when the sheep were starved for a period before treatment greater than 24 hours.

The mean regression lines of the changes in blood copper concentrations, the numbers of sheep used, and the copper dosage administered are shown in Table 4.5 and Table 4.6.

In the sheep that received 100 mg of the high concentration formulation, the liver enzyme activity concentrations in serum did not rise beyond the normal limits in any sheep. In these same sheep, using the liver biopsy results, 54% of the administered dose was present in the liver after 7 days.

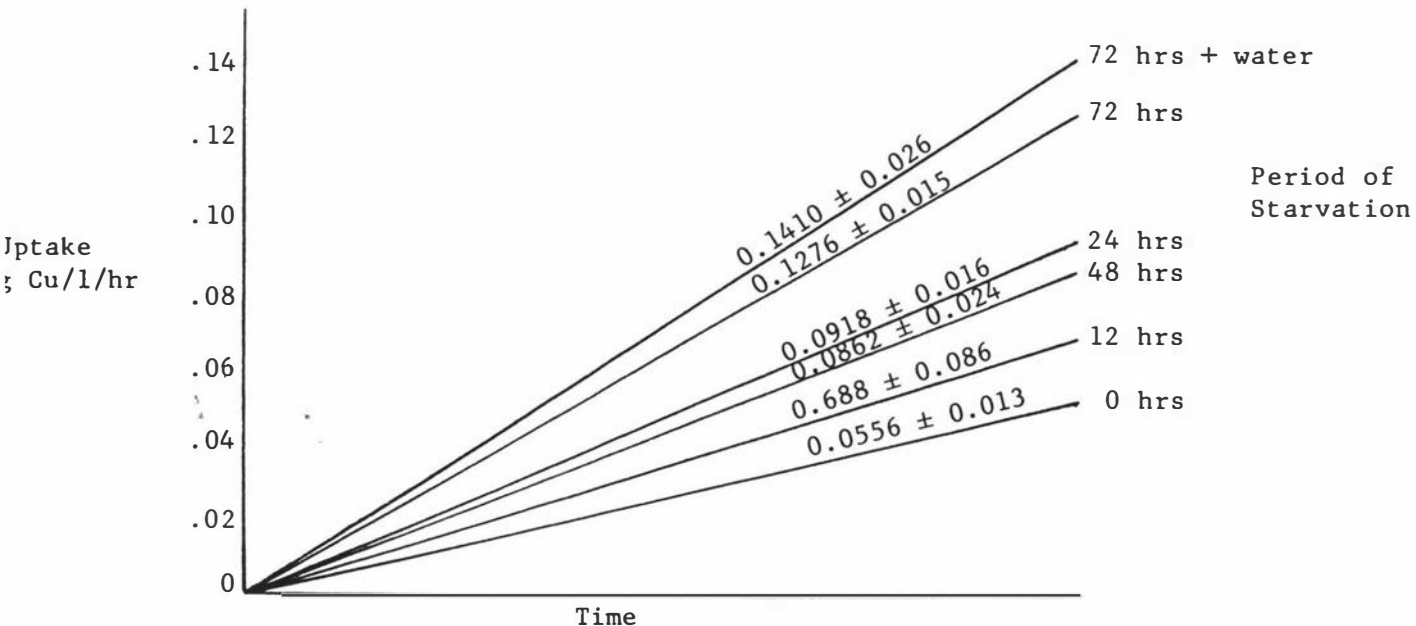
There was a statistical significance in the uptake rate of copper between those sheep that were starved for 48 hours and those starved for only 12 hours or not starved at all (Table 4.6).

Treatment	No. sheep	Dose rate	Uptake mg Cu/l/hr
Heat + 50mg	2	4 mg/kg	.0299
Oral chelate	4	33 mg/kg	.0210
CuO needles	4	2.86 gm	-.0093
Low conc form	4	50 mg	.0154
High conc form	5	50 mg	.0197
High conc form	6	100 mg	.0219
High conc + water	5	50 mg	.0356
Starve 48 hrs	2	2 mg/kg	.0842

Table 4.5: Mean uptake rates of copper in sheep administered different copper therapeutic agents.

Treatment	No. sheep	Dose rate	Uptake mg Cu/l/hr	F
Starve 72hrs	5	2 mg/kg	.1276	B
Starve 72 + H2O	4	2 mg/kg	.1410	B
Starve 48 hrs	5	2 mg/kg	.0862	AB
Starve 24 hrs	5	2 mg/kg	.0918	AB
Starve 12 hrs	5	2 mg/kg	.0688	A
Starve 0 hrs	4	2 mg/kg	.0556	A

Table 4.6: Mean uptake rates of copper in sheep administered copper calcium edetate after being subjected to different periods of starvation.



Supplement to table 4.6: Graph of mean uptakes of copper in sheep administered copper calcium edetate after being subjected to different periods of starvation.

DISCUSSION

For the purposes of this discussion, the sheep subjected to the various treatments can be classed conveniently into three groups of experiments.

The first group of experiments includes those sheep subjected to heat and those sheep treated orally with copper edetate and copper oxide needles. The copper oxide needles are slowly absorbed (Dewey, 1977) and so did not affect blood copper concentrations. The liver uptake was similarly slow as shown by the low serum enzyme activity. This supports the work of Ellis (1979) which showed that the uptake of copper from copper oxide needles was slow and appeared non-toxic to sheep.

The oral copper edetate dose is also non-toxic and yet it has an uptake rate similar to that of 50 mg of copper calcium edetate given as the low concentration formulation by subcutaneous injection. However only an estimated 7.6 mg of this oral dose was retained in the liver, and this is approximately 20% of the amount expected to be retained after a single recommended dose of parenterally administered copper calcium edetate. Nevertheless this high retention rate for orally administered copper probably resulted from the low concentrations of the inhibiting elements molybdenum and sulphur (< 0.2 ppm and < 0.07 % respectively). These latter concentrations are not typical of most New Zealand pastures on which copper deficiency is diagnosed. Sheep given this same dose rate of oral copper edetate, and on the same diet, but also given a daily supplement of 50 mg of molybdenum and 100 mg of sulphur did not demonstrate any change in liver copper concentration (Farquharson, unpublished data).

The uptake rate of the copper in the sheep in the hot environment was almost double that of a similar dose administered to sheep in a normal environment (Table 4.5). This increase was probably the result of two mechanisms. The skin temperature of these animals probably increased the subcutaneous blood supply which in

turn enhanced the absorption rate (Harvey, 1970). The other factor possibly responsible for the increased uptake was partial starvation, as these sheep had a greatly reduced feed intake over the two days previous to the copper supplementation. The mechanism of starvation is described later in the text.

The second group involved those sheep that received the different formulations of copper calcium edetate given parenterally. The lowest uptake rate occurred in the group that received 50 mg of copper as copper calcium edetate in the low concentration formulation. Those sheep that received 50 mg of copper in the high concentration formulation of copper calcium edetate, and those that received 100 mg of copper in this same formulation had similar mean uptake rates of 0.0197 and 0.0219 mg Cu/litre/hour respectively. This was only slightly higher than that obtained following 50 mg of copper in the low concentration formulation and this contained PVP which delays drug absorption (Ballard & Nelson, 1970). The high concentration formulation contained copper calcium edetate at double the concentration of the low concentration formulation. The retarded absorption of the concentrated suspension was most likely the result of a compact poorly soluble deposit formed subcutaneously (Ober et al., 1958). The effect of concentration on absorption in this experiment was comparable to the retarding effect of PVP. When the new formulation was diluted with its own volume of water, the uptake rate almost doubled. This increase was probably due to the less concentrated suspension presenting a greater surface area to the absorptive capillary bed (Yeoman, 1977), to its lower viscosity (Dowrick, 1980), and to more copper being present in solution in a more dilute suspension (MacDiarmid, 1983). The higher uptake rate would greatly enhance the toxicity of copper.

The third group of experiments comprised those sheep which had been subjected to starvation, but had all received the same dose rate of the low concentration formulation at 2 mg Cu/kg bodyweight. The uptake rate in both groups of sheep that were starved for 48 hours was almost identical. However in the control sheep that were not

starved the uptake rate was 0.0556 mg Cu/litre/hour, which exceeds the 0.0219 of the group receiving 100 mg of the new formulation. The control group was on pasture before the supplementation with copper, and then had access to hay during the hourly bleedings. These sheep did not eat their food on offer and therefore were fasting from the time of the copper injection. Unfortunately no parameters were measured at the completion of bleeding to confirm this effect.

The bodyweight losses and the blood glucose depression were typical of fasting ruminants as reported by Kirton et al., (1965), and by Bouchat et al., (1980).

Liver protein metabolism alters during fasting (Aronson, 1980); the liver losing up to 40% of its total weight over 72 hours (Furner & Feller, 1971). Most of this loss is protein. In these experiments after 72 hours of fasting, plasma protein had increased by approximately 10%, whereas plasma albumin had increased by approximately 70%. This can be explained by the fact that albumin is the smaller protein molecule and perhaps permeated through the hepatocyte cell wall more readily during times of increased demand for hepatic protein.

The increase in blood copper concentration during translocation of copper from the depot site to the liver may have resulted from any or all of three phenomena. These are, an increased blood supply at the site of deposition, an increase in the plasma protein that is the carrier protein (for copper it is albumin), or the liver receptor sites may not immediately accept the copper ions from the protein carrier complex. It is difficult to imagine that subcutaneous vascularity would alter in fasted animals when there was no variation in environmental temperature, or in local irritation at the depot site. Total blood albumin had increased dramatically and it can be assumed that hepatic protein metabolism was altered. In copper toxicity there is increased hepatic damage resulting from the increased copper content of hepatocytes as copper in the blood is presented to the liver at a higher concentration (Chapter 3). During

the accumulation phase of potential copper toxicity, there is an increase in hepatocyte copper content. It is proposed that during periods of starvation copper is presented to the liver in the copper-albumin complex at higher concentrations than in animals that have not been subjected to starvation, and therefore there is a more rapid accumulation of copper in the hepatocyte.

The uptake rate of copper in those animals not starved, and which were used as control sheep in the starvation experiment, was greater than expected. These sheep were penned for the experiment after grazing pasture and may not have eaten the hay provided. Therefore by the tenth hour of blood collection, some effects of starvation may have influenced the uptake rate of copper. Because of the difficulty in controlling all the variables in this experiment it would appear inadvisable to compare results from experiments carried out at different times.

The greater than two-fold increase in uptake rate of copper in sheep starved for 72 hours, when compared to sheep that were not starved, suggests that translocation rate is important when assessing factors that may potentiate copper toxicity. Therefore in developing new copper therapeutic compounds and their route of administration it is essential to determine the translocation rate of the copper and also to identify those factors that may alter the translocation rate.

From this data it is suggested that sheep should not be injected with copper calcium edetate at dose rates exceeding 1 mg Cu/kg bodyweight if they have been deprived of feed and water for 24 hours or more. Better still, they should not be deprived of either about the time of copper administration.

CONCLUSION

The toxicity of copper calcium edetate, after injection into sheep, may be enhanced when the uptake of copper from the depot site to the bloodstream is elevated. This increase may occur either when sheep are placed in a hot environment or when the concentration of the parenteral copper product is reduced, but the total volume injected increased.

More importantly, the toxicity is greatly enhanced if sheep have been fasted. Sheep that have been deprived of food and water for 24 hours or longer should not be injected with copper calcium edetate.

CHAPTER 5: MONITORING THE EFFECT OF COPPER SUPPLEMENTATION ON
ANIMALS GRAZING COPPER SUFFICIENT AND COPPER DEFICIENT FARMS.

INTRODUCTION

In the past, the administration of copper to domestic animals has often been based on inadequate information. There are now a number of ways in which the copper status of a farm and its animals can be assessed accurately, and from this data, the best advice on supplementation can be given. It is also necessary to appreciate the effect of copper therapeutic agents on animals of sufficient and deficient copper status. Because of the potential effects of a number of management, climatic, feeding and geographical factors, this is best monitored in animals grazing under typical farm conditions.

For the purposes of this study, two farms were selected. On farm A, copper deficiency had been regularly diagnosed in both sheep and cattle. This was indicated by a low but regular occurrence of swayback in lambs, and poor growth and reproductive performance in cattle. The cattle also showed the classical signs of coat colour change (achromotrichia).

On farm B, copper deficiency had never been diagnosed in sheep, but young cattle showed signs of copper deficiency in some years. In other years, the copper levels in cattle, as determined by regular liver biopsy, were adequate, and no clinical signs of copper deficiency were apparent. Three years prior to the commencement of the present study, this farm had been topdressed with copperised superphosphate at a rate of 5 kg copper per hectare. The apparent need for periodic copper supplementation of cattle on this farm made it a valuable property to monitor as it is typical of many New Zealand North Island sheep and beef cattle properties on which copper deficiency occurs in cattle, but not in sheep.

To calculate the availability of copper in pasture to grazing ruminants, Suttle (1974a), has developed a formula to incorporate the influence of molybdenum and sulphur on copper availability. This formula is:

$$A = 0.075 - 0.030 \text{ Mo} - 0.0134 \text{ S}$$

where A is the availability of copper

Mo is in mg/kg

S is in gm/kg

and is used in this experiment to assess the amount of copper available to the sheep on these farms.

Farm Description

Farm A, the copper deficient farm, is situated in the Turakina River valley, 20 km west of Hunterville in the Rangitikei County (Figure 5.1). The farm is 634 ha in area and consists of two distinct areas. Area 1, known as Mill Block, is of 320 ha and is divided into nine major paddocks. This is rolling hill country with a soil type classified as Mangatea clay loam. This soil is derived from a consolidated rubbly sandy mudstone (Campbell, 1979). Only 500 ha of this soil type are in the Rangitikei County. The pasture on this area is improved with a high content of perennial ryegrass (Lolium perenne) and white clover (Trifolium repens).

The area of land located at the back of the farm is area 2 and known as Wallies Block. It is 314 ha in area and is poorly subdivided into four paddocks. This is steep hill country classified as Turakina steepland soil derived from a consolidated rubbly sandy mudstone (Campbell, 1979). This land which is typical of the area (with 20,000 ha in the Rangitikei County) contains poorer quality pasture species such as brown top (Agrostis tenuis), timothy (Phleum pratense), and crested dogstail (Cynosurus cristatus), and only small quantities of ryegrass and clover.



Figure 5.1: Location of the two experimental farms

The farm is situated approximately 500 metres above sea level and enjoys a regular annual rainfall of approximately 1200 mm. There is pasture growth all year round, except on the dry faces exposed to the hot dry winds in summer. This farm is run at a stocking rate of 11.0 stock units per hectare.

The "copper sufficient" farm, farm B, is located at Tangimoana, near the coast on the western boundary of the Manawatu County (Figure 5.1). The farm is 1189 ha in area subdivided into 39 paddocks, and has a stocking rate of 9.4 stock units per hectare. The farm is coastal sand country of the Hokio-Waitarere association (Cowie, 1958), which makes up approximately 100,000 ha of this coastal strip of country. The yellow-brown sand is derived from wind-blown coastal sand of predominantly feldspar and quartz. This soil is very low in clay and organic matter. The area is mainly sand plains, with some sand dunes (Cowie, 1977). The pasture sward contains ryegrass, Yorkshire fog (Holcus lanatus), crested dogstail, and white and strawberry clovers (Trifolium fragiferum). Although the area has a regular annual rainfall of approximately 1000 mm, the main growth period for pasture is in the winter and spring as the pastures tend to dry out in summer.

MATERIALS AND METHODS

On farm A, (to be known as the copper deficient farm), the liver copper levels of the sheep and the mineral content of the pasture were monitored over an 18 month period. Four hundred Romney sheep, comprising 100 ewe hoggets, 100 two-year old ewes, and 200 mixed-age ewes, were individually identified using numbered ear tags. Eight visits were made to this farm.

On farm B, (the copper sufficient farm), the liver copper levels of the sheep and the mineral content of the pasture were monitored four times over a 12 month period. One hundred Romney ewe hoggets were individually identified and used for this purpose.

At the initial visit to each farm, soil samples were collected from the paddocks to be grazed by the trial sheep. The soil copper was extracted using 2N hydrochloric acid (Fiskell, 1965), and the copper content estimated using atomic emission spectrometry.

On farm A the sheep grazed seven paddocks. Pasture samples from these paddocks were collected for analysis on twelve occasions over the 18 month period of the trial. On farm B pasture samples were collected from the paddocks in which the identified sheep grazed, and these samples were collected on seven occasions. In each case the pasture samples were digested in nitric acid and analysed for mineral content using atomic emission spectrometry (Appendix II).

At each farm visit the tagged sheep were drafted out and weighed. Four sheep in every group of 100 were selected at random and liver biopsy samples and blood samples were collected. These samples were analysed for copper content (Appendix I & II). In addition the blood samples were analysed for PCV, haemoglobin content and plasma protein content, and the MCHC was calculated.

At the initial visit to each farm, half of the sheep in each group were injected with 50 mg of copper calcium edetate¹, contained in a 2 ml volume. On farm A these treated animals were given a further 2 ml of copper calcium edetate, 13 months after the first injection.

Eleven months after the start of the trial, the fleece weights were recorded at shearing from some of the identified animals on farm B.

The F-ratio was the test statistic applied to an analysis of variance command, to determine significance.

RESULTS

On both farms the concentration of extractable copper in the soil was similar. Amounts ranged from 2.00 ppm Cu to 16.98 ppm Cu, and the range is similar to levels recorded in many New Zealand soils (Wells, 1957). There was a positive correlation of 0.994 between soil extractable copper and pasture copper on farm B (the copper sufficient farm) (Figure 5.2). The data in Tables 5.1 and 5.2

¹ Coprin Multidose, Glaxo (N.Z.) Ltd.

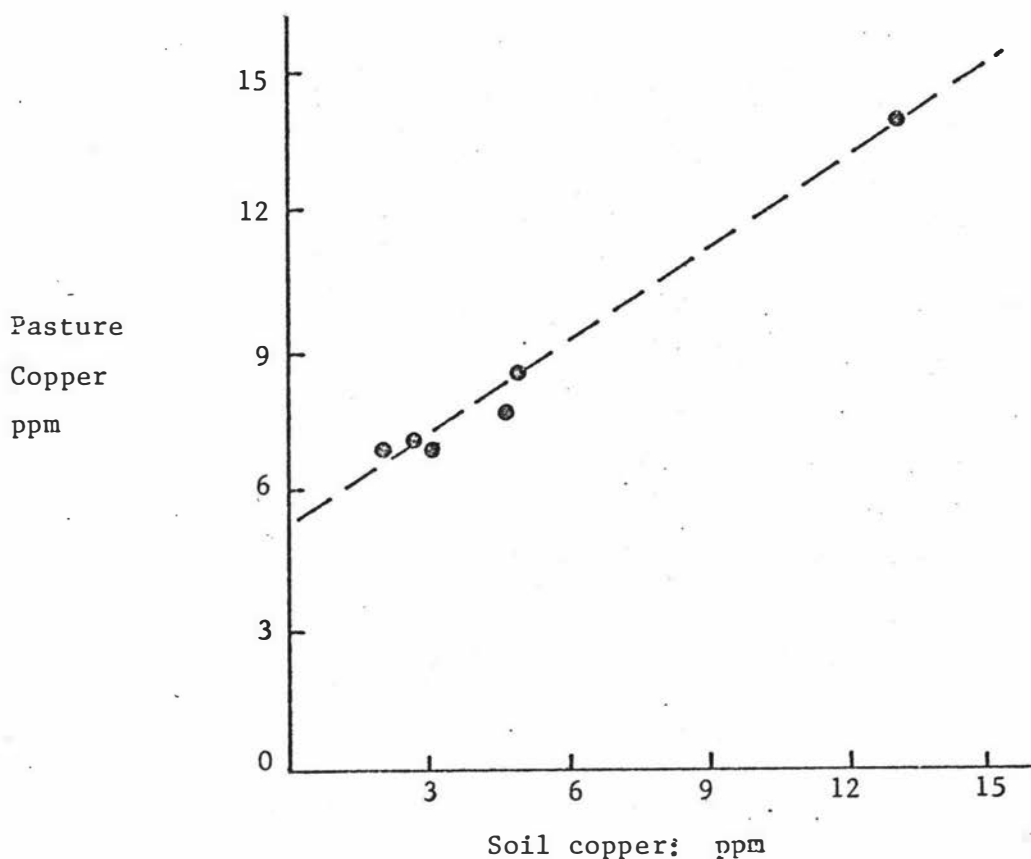


Figure 5.2: Relationship between soil copper and pasture copper on the copper sufficient farm

represent the average mineral content of the pasture from the two areas of the copper deficient farm.

On both areas of the farm the pasture copper levels as estimated from the samples analysed, were lowest over the winter period and highest in the summer, whereas the amounts of molybdenum and sulphur were highest in the early spring period, with molybdenum as high as 4.74 ppm D.M., and sulphur as high as .32% D.M. (Figure 5.3). The lowest molybdenum levels measured were 1.41 ppm D.M., with a mean of 3.13 ppm D.M.

Dates	Cu ppm	Mo ppm	S %	Fe ppm	Zn ppm	Mn ppm	S/McL ppm
12.6.81	4.36	1.41	.14	5920	39.3	137	5
25.6.81	4.08	2.70	.15	3412	43.4	221	5
13.7.81	5.43	2.85	.20	6485	52.4	296	6
25.9.81	6.52	3.29	.23	6552	85.7	322	7
13.10.81	6.40	3.69	.22	3449	54.3	309	7
20.11.81	5.34	2.35	.22	1428	41.1	246	6
3.3.82	8.91	3.60	.26	1512	77.8	132	8
25.5.82	3.68	2.02	.17	1529	86.1	273	6
15.6.82	6.79	3.50	.23	1612	58.1	250	7
16.7.82	5.78	3.81	.22	2201	61.6	191	7
Mean	5.73	2.92	.20	3401	60.0	238	6.4

S/McL is the estimated copper content of pasture to meet dietary requirements (Suttle & McLauchlan formula).

Table 5.1: Mineral content of pastures taken from area 1 on the copper deficient farm.

Dates	Cu ppm	Mo ppm	S %	Fe ppm	Zn ppm	Mn ppm	S/McL ppm
12.6.81	7.42	2.00	.24	2501	66.0	177	7
25.6.81	6.10	2.43	.19	2565	36.6	116	6
13.7.81	6.85	2.32	.22	4309	60.5	141	6
13.10.81	6.84	4.74	.36	1668	49.0	127	10
20.11.81	4.87	4.54	.21	367	28.7	57	7
10.12.81	7.69	2.98	.32	344	59.7	46	8
3.3.82	8.93	2.83	.29	422	56.4	98	8
25.5.82	7.10	2.06	.23	545	56.6	112	6
15.6.82	9.30	4.72	.28	1451	52.5	103	9
16.7.82	7.49	4.69	.26	1671	42.0	81	8
22.10.82	11.13	3.15	.34	2877	54.5	141	9
Mean	7.61	3.31	.27	1704	51.1	109	7.6

S/McL is the estimated copper content of pasture to meet dietary requirements (Suttle & McLauchlan formula).

Table 5.2: Mineral content of pastures taken from area 2 on the copper deficient farm.

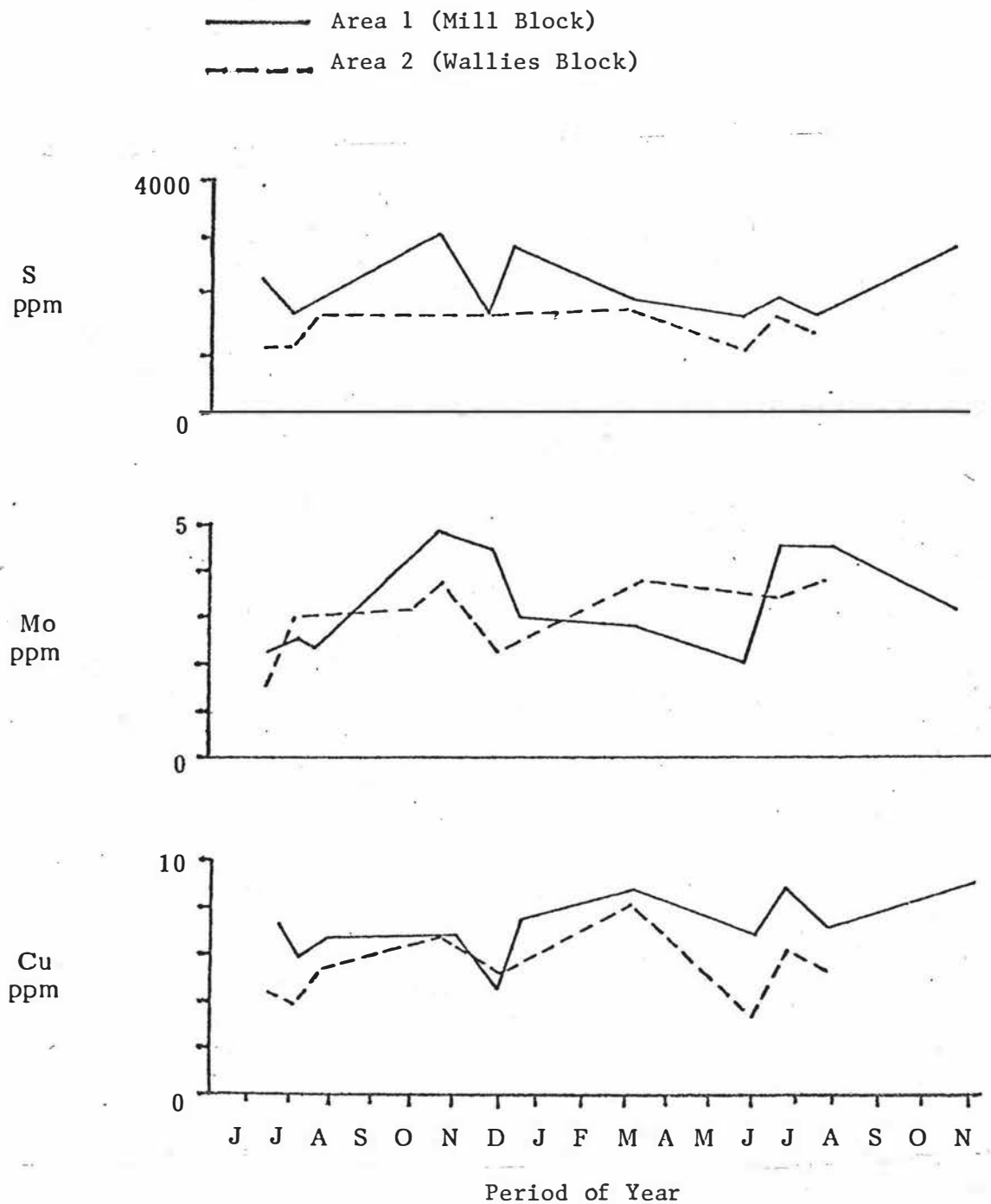


Figure 5.3: Copper, molybdenum and sulphur content of pastures on the two areas of the copper deficient farm.

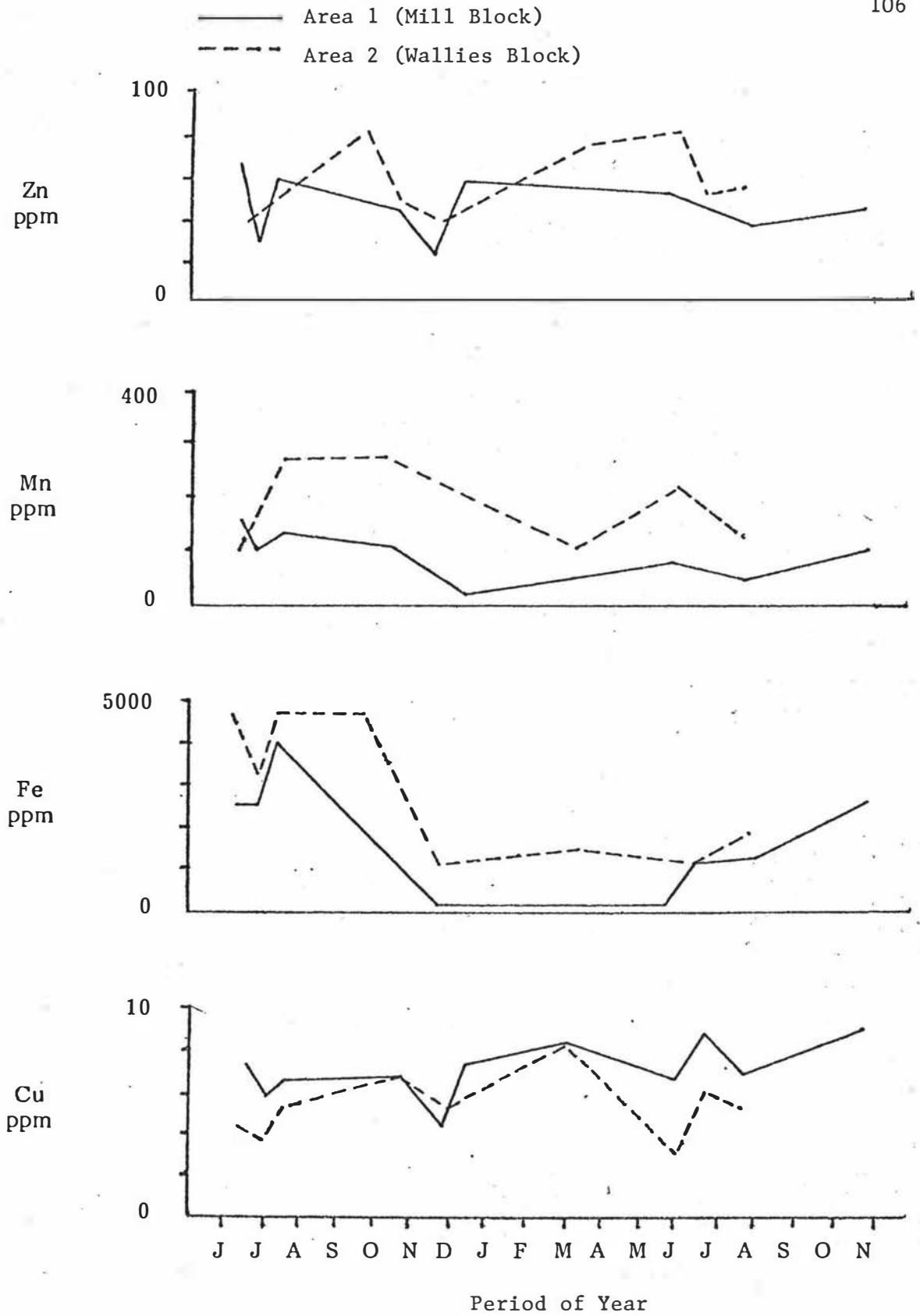


Figure 5.4: Copper, iron, zinc and manganese content of pastures on the two areas of the copper deficient farm

The major difference between the pasture mineral content of the two areas of farm A is the concentration of the soil-contaminating elements (iron, zinc, and manganese) (Figure 5.4); elements which may inhibit the uptake of copper by the animal. On area 2 the concentrations of these elements were higher than those on area 1, at all times of the year. Over the winter-early spring period, the concentrations of these elements in pasture rose to almost three times the content of the same elements in pasture from area 1. By contrast, the copper sufficient farm provided adequate copper (Suttle & Mclauchlan, 1976) throughout most of the year, with a mean pasture content of 8.97 ppm D.M. (Table 5.3). Molybdenum concentrations recorded were below 1.31 ppm D.M., and the sulphur levels averaged .23% D.M. Iron, zinc, and manganese had mean concentrations of 1662, 52.3, and 131 ppm D.M. respectively. Their influence on copper uptake was not known but probably at the concentrations recorded they would not influence the copper absorption significantly.

There were no significant differences between bodyweights of the copper treated and the control groups of animals (Tables 5.4 and 5.5).

On both farms the copper-treated sheep had a higher mean liver copper concentration than the untreated sheep (Tables 5.6 and 5.7). There was some variability in the means of the liver copper concentrations due to individual animal variation, as on each occasion a random selection of sheep from each group was used. On the copper deficient farm the liver copper levels fell rapidly during the winter, but by early summer the difference between the treated and the untreated groups was not great; 84 ppm and 67 ppm Cu D.M. respectively. However on the copper sufficient farm the liver copper levels showed little variation throughout the year. Also on this farm the copper treated sheep did have an overall higher liver copper concentration initially, and they maintained this higher concentration throughout the year.

Dates	Cu ppm	Mo ppm	S %	Fe ppm	Zn ppm	Mn ppm	S/McL ppm
8.10.81	14.11	.53	.28	574	36.5	203	6
	7.19	1.23	.12	6407	38.5	223	5
	6.99	.59	.24	712	38.0	185	6
4.11.81	6.84	.74	.18	1931	34.9	122	5
	8.59	.76	.22	808	40.9	131	6
	7.70	.70	.21	1053	82.7	160	6
21.1.82	9.22	.42	.21	517	71.3	73	5
	2.78	.21	.28	552	28.2	76	6
	3.09	.11	.24	204	37.3	59	5
9.6.82	14.69	.28	.31	3006	94.4	93	5
	11.36	1.31	.19	2534	66.1	159	6
	15.10	1.00	.27	1645	59.4	85	7
Mean	8.97	.66	.23	1662	52.3	131	5.7

S/McL is the estimated copper content of pasture to meet dietary requirements (Suttle & McLauchlan formula).

Table 5.3: Mineral content of pastures taken from the copper sufficient farm.

Dates	Bodyweight : kg					
	Hoggets		Two Year Old		Mixed Age	
	No Cu	Cu	No Cu	Cu	No Cu	Cu
22.4.81	29.0	29.8	44.8	46.2	55.0	48.8
	± 0.65	± 0.71	± 0.54	± 0.68	± 0.45	± 0.63
21.5.81	32.6	33.6	46.9	47.0	53.4	51.1
	± 0.63	± 0.69	± 0.42	± 0.53	± 0.46	± 0.48
10.8.81	36.2	36.0	46.8	48.2	53.6	52.4
	± 0.62	± 0.67	± 0.50	± 0.66	± 0.49	± 0.52
20.11.81	39.9	39.5				
	± 0.57	± 0.61				
10.12.81			50.5	51.2	57.8	56.1
			± 0.51	± 0.73	± 0.57	± 0.63
26.5.82	49.4	49.8	50.7	51.1	55.7	54.5
	± 0.67	± 0.91	± 0.65	± 0.90	± 0.66	± 0.71
8.12.82	50.0	49.4	50.6	51.8	53.3	53.1
	± 1.22	± 1.07	± 0.76	± 0.92	± 0.99	± 0.85
Weight change	21.0	19.6	5.8	5.6	-1.7	4.3

No Cu = untreated

Table 5.4: Bodyweights of sheep grazing the copper deficient farm.

Dates	Bodyweight : kg	
	Two Year Old	
	No Cu	Cu
17.12.81	54.0	52.3
10.2.82	59.4	58.2
5.5.82	56.5	56.0
29.11.82	55.1	52.2
Weight change	1.1	-0.1

No Cu = untreated

Table 5.5: Bodyweights of sheep grazing the copper sufficient farm.

Date	Liver copper content : ppm D.M.								
	Hoggets		Two Year Old Mixed Age				Flock		
	No Cu	Cu	No Cu	Cu	No Cu	Cu	No Cu	Cu	Cu
22.4.81	58	-	176	176	37	37	77	77	
	± 19.8		± 71	± 71	± 12.4	± 12.4			
21.5.81	99	-	165	264	87	134	114	160	
	± 75		± 17.3	± 39.3	± 28.8	± 38.7			
10.8.81	80	-	73	59	30	92	59	81	
	± 28.8		± 33.5	± 19.0	± 8.2	± 40.7			
14.10.81	65	-	210	-	24	83	67	84	
	± 30.1		± 33.2		± 9.8	± 14.5			
20.11.81	-	-	169	83	-	-	169	83	
			± 46.9	± 14.7					
10.12.81	25	-	-	-	37	22	31	22	
	± 2.7				± 12.0	± 3.8			
26.5.82	34	-	49	88	37	17	39	53	
	± 17.1		± 13.0	± 29.5	± 25.0	± 8.5			
8.12.82	19	69	45	50	39	31	34	50	
	± 4.5	± 30.6	± 14.0	± 14.5	± 11.0	± 6.5			
Mean	55	69	128	90	42	70	62	77	

No Cu = untreated
 - = no sample

Table 5.6: Liver copper content of sheep grazing the copper deficient farm.

was no correlation between the blood copper content and the liver copper content, on either farm. Those sheep supplemented with copper did not have higher blood concentrations, and there was no difference in the blood copper concentration between those sheep grazing the copper sufficient farm and those sheep grazing the copper deficient farm.

The blood parameters of packed cell volume, haemoglobin content, MCHC, and plasma protein stayed within normal limits at all times and were not affected by the liver copper status of the animal, or the farm on which the sheep grazed.

<u>Liver copper: ppm D.M.</u>		
Two Year Old		
<u>Dates</u>	<u>No Cu</u>	<u>Cu</u>
4.11.81	331	-
17.12.81	429	530
10.2.81	480	627
5.5.82	627	530
29.11.82	553	367
Mean	440	514

No Cu = untreated

- = no sample

Table 5.7: Liver copper content of sheep grazing the copper sufficient farm.

Of the forty fleeces weighed on the copper sufficient farm, the copper treated sheep had a mean fleece weight of 4.15 kg, compared to the control sheep which averaged 4.29 kg. This difference was not significant.

DISCUSSION

Due to the high organic matter content and the high clay content of the soil, the amount of soil copper extracted using 2N hydrochloric acid bears little relationship to the total amount of copper present in pasture (Robson & Reuter, 1981). The data recorded on these farms support this belief, particularly on farm B, the copper deficient farm. However on the copper sufficient farm the correlation between extractable soil copper and pasture copper content was surprisingly high ($r = 0.994$). This high correlation is probably due to the nature of the soil on this farm. The soil is yellow brown sand which is low in both organic matter and in clay

content. Such soils are known to have more freely available copper i.e. in an unbound state (Thornton, 1979; James & Burrow, 1981).

However, in the light of the mineral analysis of pasture, the reason for the presence of copper deficient animals on farm A (the copper deficient farm) and not on farm B (the copper sufficient farm) appears more obvious. The copper sufficient farm provided adequate concentrations of pasture copper throughout the year (as judged by the Suttle & McLauchlan formula, 1976). There were also lower levels present of those elements known to inhibit the absorption of copper. On farm B, the other elements which are believed to inhibit copper uptake, namely, iron, zinc, and manganese were all at low levels in the pasture throughout the year. However according to the work by Mills, (1980); Reynolds, (1979), and Grace, (1973), iron, zinc, and manganese probably had little effect on copper absorption. Applying this data to the graph of Cornforth (1980) from the formula of Suttle & McLauchlan (1976), in only two paddocks was the pasture unable to provide adequate copper for sheep (Table 5.3).

On the copper deficient farm there was considerable variation in the mineral content of pasture between paddocks. On area 1 (Mill Block), the pasture copper concentration was adequate throughout the year (3.68 - 8.91 ppm D.M.). Further, the inhibiting elements remained low throughout the year, except for molybdenum which rose in late spring in one year and in winter the following year (Figure 5.3). When this data is examined in the light of the Cornforth graph, 77% of the grazed area was able to supply adequate pasture copper for sheep.

Area 2 of farm A, known as Wallies Block, had low copper concentrations in pasture throughout the year. These dropped to their lowest concentrations during winter, while conversely the molybdenum and sulphur concentrations rose to their highest during this period. The other elements which depress copper absorption by animals were also at their highest level during the winter. This is probably the result of soil contamination of this area; 50% of which

faces south. This aspect, together with the high rainfall and heavy continual grazing generated the mud which caused pasture contamination. Again if the Cornforth modification of the Suttle & MacLachlan formula is applied to this data, then only 34% of these paddocks were able to supply adequate copper for sheep. Unfortunately this is the area of the farm on which the main ewe flock was wintered. At this time of the year the demand for copper by the ewe is highest (Grace, 1983) and the availability of copper the lowest. Further these paddocks are large and contain grass species of low digestibility which is likely to further lower the availability of absorbable copper.

The concentration of copper relative to the concentration of the other inhibiting elements on the copper deficient farm suggests an explanation for the regular appearance of swayback lambs and the signs of copper deficiency in the cattle. It must also be noted that cattle have a 50% higher requirement than sheep for copper in pasture (Suttle, 1981b).

However from the data collected from the copper deficient farm (Tables 5.1, 5.2, & 5.6), it does appear that an annual supplementation of 50 mg of copper as copper calcium edetate was sufficient to prevent the appearance of copper deficiency in sheep. These data also confirm the importance of knowing the levels of pasture copper and other minerals on various parts of the farm where copper deficiency exists. With such information greater utilization of copper sufficient areas may be made during the periods when the copper requirements of grazing animals are high.

There was no significant difference in the bodyweights between the treated and untreated sheep on each farm, and between the sufficient and deficient farms. This reaffirms that weight gain response trials are probably of little value in assessing copper status of animals. Only Hogan et al. (1971), Whitelaw et al. (1979), and Whitelaw et al. (1980) have been able to measure a significant weight response to copper supplementation. However the latter two

trials were carried out using lambs, and the first trial was with sheep on a diet which was very high in molybdenum. No significant reduction in bodyweights of sheep was found on the copper deficient farm even though some of the non-treated ewes produced lambs that subsequently developed enzootic ataxia.

Whitelaw *et al.* (1979) also noted significant differences in fleece structure and in wool characteristics in their trial. However in the trial on the copper sufficient farm there was no difference in either of these two features or in the weight of wool shorn from each ewe. None of the sheep showed signs of copper deficiency or had liver or blood tissues which had low concentrations of copper at any stage of the trial.

The liver copper content of the sheep on the respective farms was different. On the copper deficient farm the liver copper concentrations fell during the winter, rose slightly during the summer, only to fall again during the next winter. On this farm, liver copper concentrations declined rapidly after treatment, but maintained a slightly higher concentration throughout the rest of the year in comparison with the control animals. Obviously during the winter there is a very high demand for stored liver copper to maintain copper homeostasis. This is due in part to the higher demand for copper in late pregnancy (Suttle, 1981b), and from the low availability of pasture copper, due to the inhibiting factors already discussed.

The degree of absorption of copper from the diet can be calculated from the results of the pasture analysis, the liver analysis of the untreated animals on both farms, and from assuming that the copper requirements for a ewe for maintenance are 0.01 mg Cu/kg bodyweight/day (Grace, 1983). Using the criteria above, on the copper sufficient farm, 4.7% of dietary copper was absorbed, whereas on the copper deficient farm 6.4% of dietary copper was absorbed. The copper deficient farm had pasture high in inhibiting elements which affected the absorption and retention of pasture copper. The

sheep on the copper sufficient farm however, had high liver copper levels and maintained these at a relatively constant level. In addition, this latter farm had pastures of adequate copper levels and a lower content of inhibiting elements. This difference in intestinal absorption of copper would suggest that sheep are able to control their copper absorption, which supports the work of Neethling et al., (1968), and of Hill et al. (1969). A mechanism may operate whereby the animal is able to control the absorption of intestinal copper, regulate the excretion of copper, or control the function of both processes.

The haematological results were similar to those reported for normal sheep. In addition, the blood copper concentration was within the normal range; the sheep which were treated with parenteral copper had liver copper concentrations similar to those found in the untreated sheep. In all cases the blood copper concentrations bore no relationship to the storage concentrations of copper in the liver. This lack of correlation suggests that unless an animal is hypocupraemic, then blood samples are of little value in assessing its copper reserves.

In the sheep which regularly grazed farms A and B, there were marked differences in the liver storage concentrations of copper. On farm B, which was copper sufficient, the sheep maintained high liver concentrations of copper throughout the year (331 - 627 ppm D.M.). Furthermore, on this farm the elements which may have prevented the uptake and retention of copper by the animals were at low concentrations in the pasture. In contrast, the liver copper concentrations of the sheep grazing the copper deficient farm showed a substantial variation throughout the year. The lowest liver copper concentration occurred in the winter when pasture copper concentrations were low and the content of inhibiting elements high. This suggests that the measurement of copper requirements for animals may be evaluated most effectively in the winter, particularly as this is a time when advice on copper supplementation may be required.

It would appear that on a copper sufficient farm, similar to the one studied in this experiment, the copper status assessed from the animals and the plants, may be confirmed at any time of the year. However on a potentially copper deficient farm, it is important to know the status of the animals prior to the period of greatest demand, that is, the winter. Assessment of liver copper stores, and of pasture mineral content, will assist in making a decision about any need for copper supplementation at this time of the year.

The supplementation of the sheep on the copper deficient farm with 50 mg of copper, as copper calcium edetate, was effective in elevating liver copper concentrations immediately after injection, although these concentrations steadily decreased during the ensuing year. The timing of the injection was important as it ensured that the animals had high copper stores during the period of greatest copper demand and lowest dietary copper availability. There were no lambs with enzootic ataxia born to the ewes supplemented with copper. However there were three known cases of swayback in lambs born to the untreated ewes.

Conversely, in the ewes grazing the copper sufficient farm and injected with 50 mg of copper as copper calcium edetate, the liver copper stores rose immediately after the injection and remained at this elevated level throughout the duration of the experiment.

CONCLUSION

Regular monitoring of the mineral content of pastures and the copper concentrations of animals using liver biopsies, helps in understanding the dynamic status of copper on that farm. From this information, supplementation procedures can be considered.

Liveweight changes in response to copper supplementation are not an effective method of diagnosing copper deficiency. Similarly blood copper concentrations do not indicate how adequate are the copper stores of the animal.

Supplementation of sheep with 50 mg of copper as copper calcium edetate was effective in elevating liver copper storage levels. It is important that the liver copper stores are elevated prior to the periods of high copper demand.

CHAPTER 6: THE DIFFERENCES IN THE ABSORPTION OF COPPER AMONGST FOUR
DIFFERENT BREEDS OF SHEEP IN NEW ZEALAND.

INTRODUCTION

There are considerable differences between breeds of sheep in their ability to absorb copper from the diet. This interesting phenomenon has been reported by several workers, experimenting with British breeds of sheep (Wiener & Field (1969), Wiener et al. (1969), Wiener & Field (1970), Wiener, Herbert & Field (1976), Wiener, Wilmut & Field (1978), and Herbert, Wiener & Field (1978)). Luke & Weirman (1970) and van der Berg et al. (1983) have also shown differences between the European breeds of sheep. Any variation between breeds diminishes as the molybdenum content of the diet increases (Suttle, 1981b), and it would seem the rumen is the site at which interference in copper absorption occurs (Wiener, 1980). No such comparative work on the uptake and retention of copper has been done using the breeds of sheep commonly farmed in New Zealand.

In order to establish whether any significant differences in copper absorption by sheep of different breeds occurred in New Zealand, four breeds of sheep were selected. All of these have played a major role in our sheep industry. Each of these breeds was considered to be "genetically diverse", according to the thesis of Ryder (1964) that proposed the probable lines of evolution of British breeds of sheep (Figure 6.1). The four breeds selected were the Border Leicester, Merino, New Zealand Romney, and Suffolk.

The aim of the experiment was to reduce liver copper reserves of the sheep to a stage when the majority had copper concentrations below 100 ppm Cu D.M. The sheep were then grazed on a farm where the availability of copper in the pasture was known to be high. In fact, the farm that was selected had a history of copper toxicity in sheep on several occasions. Following grazing on this farm to allow significant increases in liver copper content (approximately three months), each sheep was dosed with copper parenterally and the liver

During the stage of liver copper depletion, all the sheep were housed and fed on a diet of hay containing 6.2 ppm D.M. of copper, and allowed free access to water. All sheep were dosed daily with 20 ml of a solution of ammonium molybdate and sodium sulphate, containing 50 mg molybdenum and 450 mg of sulphur, to help reduce the liver copper reserves (Suttle & Field, 1968b). This treatment continued for two months after which time the second liver sample was taken.

From the results of the analyses of the copper concentration of the second liver biopsy, the sheep were divided into two groups. Those sheep with liver copper levels below 150 ppm D.M. were maintained on the hay diet and the daily dosing with ammonium molybdate and sodium sulphate was continued. The remaining sheep with liver copper concentrations above 200 ppm D.M. were given a low copper diet similar to that described by Suttle & Field (1968b) (Table 6.1).

On chemical analysis this diet contained 1.3 ppm Cu D.M. Each sheep received a daily allowance of 1.0 kg of this basal low copper diet and in addition received a daily dose of 20 ml of the solution containing ammonium molybdate and sodium sulphate. This treatment of the two groups of sheep continued for 8 weeks.

When the third series of liver samples were analysed, it was found that instead of the anticipated decrease in copper concentrations, the latter had actually risen. At this stage no explanation could be given, but the possibility of copper being absorbed from bedding straw eaten by these sheep, or a high copper content in the drinking water, could not be excluded.

In an attempt to overcome this lack of liver depletion, all sheep were moved, and grazed on a known copper deficient farm for six months. This grazing period was from June to December. At the end of this time liver biopsy samples from each sheep were analysed for copper content and were found to have fallen below 150 ppm D.M. in

Whole oats	20 %
Oat husks	17 %
Starch	17 %
Sucrose	17 %
Dried skim milk powder	16 %
Urea	2 %
Peanut oil	2.5 %
Potassium bicarbonate	2 %
Sodium chloride	1.6 %
Magnesium oxide	0.5 %
Vitamin A	1000 i.u./kg
Vitamin D	140 i.u./kg
Vitamin E	36 i.u./kg
Iron	100 mg/kg
Manganese	50 mg/kg
Zinc	20 mg/kg
Iodine	8 mg/kg
Cobalt	2 mg/kg

Daily allowance 1.0 kg/sheep/day

Table 6.1: Composition of basal low copper diet
(Suttle & Field, 1968b)

all except three of the sheep. At this stage of the study, depletion of liver copper was considered to have reached a sufficiently low level to act as a baseline for subsequent copper supplementation and estimations of uptake.

All the sheep were then grazed on a farm of known high copper status, where deaths from copper poisoning had previously occurred. These sheep remained on this farm for four months before a fifth liver biopsy was taken and analysed. At this time the sheep were returned to Massey University.

Copper calcium edetate¹, at a rate of 1 mg/kg bodyweight was then injected into each sheep. The sheep were grazed on pasture containing 6.6 ppm D.M. of copper and after two weeks liver biopsy samples were again taken for copper estimations.

At the time of each liver biopsy, wool samples were collected after shaving over the biopsy site. The samples were cleaned, and analysed for copper content. Sheep bodyweights were also recorded at the time of each liver biopsy.

While the sheep were on the two farms, pasture samples were collected monthly and analysed for mineral content.

For the calculation of the absorption of copper from the diet it was assumed that the requirements for copper are 0.01mg/kg bodyweight/day for maintenance, and 4 mg for each kilogram increase in bodyweight (Grace, 1983). It was also assumed that any loss of copper from liver was used in general metabolism and any increase in liver copper was from a dietary source. Liver mass was estimated to be 1.13% of total bodyweight and contained 29.4% as dry matter (van Ryssen, 1980), and the estimated daily intake of pasture was 1.2 kg D.M./day and of the synthetic diet 0.9 kg D.M./day.

The equation used to calculate the percentage absorption of copper from the diet was:

$$\text{absorption} = \frac{mx(b+f)/2x.01 + (f-b)x4 + \{(exf)-(axb)\}1.13\%x29.4\%}{m \times y \times z} \times 100$$

¹ Coprin Multidose, Glaxo (N.Z.) Ltd.

where a = mean liver copper content at start (ppm D.M.)
 b = mean bodyweight at start (kgs)
 e = mean liver copper content at end (ppm D.M.)
 f = mean bodyweight at end (kgs)
 m = no. of days on diet
 y = copper content of diet (ppm D.M.)
 z = feed intake (kg D.M./day)

An example of the calculation for the absorption of copper from the copper high pasture is set out below:-

Mean liver copper at start	= 86 ppm
Mean bodyweight at start	= 60.2 kg
Total liver copper at start	= 86 x 60.2 x 1.13 % x 29.4 % = 17.2 mg
Mean liver copper at end	= 289 ppm
Mean bodyweight at end	= 64.9 kg
Total liver copper at end	= 289 x 64.9 x 1.13 % x 29.4 % = 62.1 mg
Copper increase stored in liver	= 62.1 - 17.2 = 44.9 mg
Copper requirements	= 112 days x 62.45 kg x .01 metabolism + 4.7 kg x 4 mg weight increase + 44.9 mg increased liver store = 133.6 mg
Dietary intake	= 1.2 kg x 112 days x 6.9 ppm = 927.4 mg
% absorbed from diet	= 133.6/927.4 = 14.3%

The F-ratio applied to an analysis of variance one-way command was the test used to determine the significance of differences.

RESULTS AND DISCUSSION

The changes in bodyweight that occurred were directly related to food intake (Table 6.3). On the two farms the sheep were given access to surplus pasture. The one Merino that lost weight on the high copper pasture farm became infected with foot-rot. All sheep lost weight when on the synthetic diet as it was not highly palatable.

The analyses of the diets used throughout these experiments are shown in Table 6.4. The straw and water were analysed as the sheep had access to these during the depletion phase, but the analysis showed their copper content was not sufficiently high to affect significantly the total dietary copper content. The results of the wool analyses are discussed in Chapter 7.

On the initial hay diet, containing 6.4 ppm D.M. of copper, (Table 6.4), the liver copper content of most sheep actually increased. Only the Merinos showed a significant decrease. The supplementation with ammonium molybdate and sodium sulphate appeared to have little effect on copper uptake. However these sheep were on a hay diet, and according to Suttle (1981a) 7.2% of dietary copper is absorbed from hay in comparison with 2.3% from summer pasture.

The analyses of liver biopsy samples are given in Table 6.2. The increase in liver copper content for sheep on the basal low copper diet was in direct contrast to the findings of Suttle & Field, (1968b), yet analysis of the diet showed similar levels in both cases, namely, 1.3 ppm Cu D.M. From the analysis, straw and water contained insufficient copper to have any influence on the increased storage levels of copper. The only apparent difference in the diets, was that the diet used by Suttle & Field was pelleted, while the diet in this experiment was fed as a powder. This difference in feed

Liver copper concentration : ppm D.M.									
Breed	Sheep no.					Change		Change	
		Hay	Basal	Def.	onhigh	Pasture	fromCu		
		1	2	3	4	copper	5	6	inject
	21	191	233	370	36	+282	318	867	+549
Border	22	39	<12	89	21	+142	163	509	+346
Leicester	23	355	347	511	55	+276	331	618	+287
	24	138	428	653	138				
	Mean					+233			+394
	37	101	129	161	76	+284	360	671	+311
Suffolk	38	112	293	327	198	+158	356	650	+294
	39	154	289						
	40	256	363	557					
	Mean					+221			+302
	51	294	499	670	147	+141	288		
N.Z.	52	185	488	606	264	+272	536	1149	+613
Romney	53	140	414	738	164	+312	476	551	+75
	54	30	22	80	15	+128	143	341	+198
	Mean					+237			+295
	63	150	<12	200	127				
	64	29	<12	<12	15	+41	56	293	+237
Merino	65	149	145	177	14	+134	148	340	+192
	66	241	246	314					
	67	29	<12	60					
	Mean					+88			+215

Basal = Basal low copper diet

Def. = 'Copper deficient' farm

Table 6.2: Copper content of liver samples from different breeds of sheep given four different diets, followed by parenterally administered copper; and the change in liver copper concentration while on the low and high copper regimes.

Bodyweight and weight changes: kgs.								
Breed	Sheep no.	Hay		Basal		Both		Farms Weight 4
		Weight 1	Diet Change	Weight 2	Diet Change	Weight 3	Change	
	21	51.4	+6.3	57.7	-5.7	51.8	+16.4	68.2
Border	22	58.6	+3.7	62.3	+1.3	63.6	+9.6	73.2
Leicester	23	46.8	+4.6	51.4	-7.3	44.1	+14.1	58.2
	24	51.4	+3.1	54.5	-8.1	46.4		
	Mean		+4.4		-5.0		+13.4	
	37	50.0	+3.2	53.2	-7.3	45.9	+28.6	74.5
Suffolk	38	47.3	+5.9	53.2	-9.6	43.6	+28.7	72.3
	39	43.2	+15.0	58.2				
	40	45.5	+8.1	53.6	-7.2	46.4		
	Mean		+8.1		-8.0		+28.7	
	51	59.5	-2.7	56.8	-9.1	47.7		
N.Z.	52	74.1	-8.6	65.5	-7.8	57.7	+20.5	78.2
Romney	53	60.0	-0.5	59.5	-8.1	51.4	+15.9	67.3
	54	62.7	-9.1	53.6	-2.2	51.4	+9.5	60.9
	Mean		-5.2		-6.8		+15.3	
	63	42.3	-5.9	36.4	-	36.4		
	64	51.5	+1.2	52.7	-7.2	45.5	-1.4	44.1
Merino	65	45.7	-0.2	45.5	-1.9	43.6	+6.4	50.0
	66	46.1	-1.6	44.5	-10.0	34.5		
	67	45.8	-1.3	44.5	-2.2	42.3		
	Mean		-1.6		-4.3		+2.5	

Table 6.3: Bodyweights and bodyweight changes of different breeds of sheep when eating different diets.

Diet	Cu ppm	Mo ppm	S %	Fe ppm	Zn ppm	Mn ppm
Hay	6.4	0.8	.14	30	19	55
Basal low diet	1.3	<0.2	.07	40	25	48
Bedding straw	2.1	<0.2	.18	24	33	11
Water	<0.01	-	tr	0.6	0.1	0.03
Copper deficient pasture	9.3	4.2	.29	2000	49	108
High copper pasture	6.9	1.1	.22	281	21	40
Massey pasture	6.6	0.9	.36	1075	45	83

Table 6.4: Mineral content of respective diets used.

texture appears to be the factor that could have caused the variation in absorption of copper for the following reasons. Inhibition of copper absorption occurs in the rumen (Suttle, 1981c; Whitelaw et al., 1982). The passage of dietary contents through to the reticulo-omasum from the rumen is dependent on particle size. Those particles of diameter less than 0.6 mm apparently pass straight through to the reticulo-omasum (Ellis et al., 1979) and on to the abomasum. The majority of the particles in the synthetic diet were less than 0.6 mm, thus the rapid passage of ingesta would leave little opportunity for copper ions to be rendered insoluble in the rumen by the thiomolybdate complexes.

The absorption of copper from the three major diets, namely, the synthetic diet, the 'copper deficient' pasture, and the 'high copper' pasture, was calculated according to the formula described previously, and by using the data from Tables 6.2, 6.3, and 6.4. These calculations gave the following absorption rates:-

synthetic diet	76.6 %
copper deficient pasture	4.8 %
high copper pasture	14.3 %

The difference in absorption rates of copper from the pasture between the copper deficient farm and the high copper farm was substantial. The copper deficient farm had pasture of a higher copper content (9.3 vs 6.9), but also it contained higher levels of the copper inhibiting elements, molybdenum, sulphur, iron, zinc and manganese. By applying the Cornforth modification of the Suttle & McLauchlan formula to these pastures, using copper, molybdenum and sulphur only; both diets would appear to have provided adequate copper. However, the mean differences between the two farms for iron, zinc and manganese were great; viz., 2000, 40 and 108 ppm respectively for the copper deficient farm, and 281, 21 and 40 ppm respectively for the high copper pasture farm. These minerals then may still have had a contributory effect.

There was also a considerable difference in the pasture quality between the two farms. The copper deficient farm had pasture consisting of poor quality species of low digestibility, for example, brown top, danthonia, and crested dogstail, whereas the pasture on the high copper farm contained species of higher digestibility, especially perennial ryegrass and white clover. The pasture of low digestibility would have spent a greater amount of time in the rumen until it was broken down into particles sufficiently small to pass on to the reticulo-omasum (Kay, 1983). This longer period of time may have allowed a greater opportunity for the inhibiting elements to be effective in restricting the uptake of copper.

The feeding of the synthetic diet resulted in a calculated absorption of approximately 76.6% of the copper. This absorption rate is very high and approximates that of the pre-ruminant lamb (Suttle, 1981b). This similarity would suggest that the components of this diet either spent a very brief period of digestion in the rumen or that they bypassed the rumen almost completely.

The differences in the absorption and retention of copper measured in this experiment justify its repetition and extension, using a greater number of animals, in an attempt to verify the preliminary results. There was no significant difference between the three British breeds i.e. the Border Leicester, N.Z. Romney, and Suffolk (Table 6.2) when considering the uptake of copper from the high copper pasture diet, and also the liver retention of copper after the parenteral supplementation with copper. The lack of breed differences may be supported from field work in New Zealand where investigations into clinical enzootic ataxia have never implicated a particular breed.

The Merino, however, differs from the three British breeds. The uptake of dietary copper was significantly lower ($P < 0.05$) in the Merino, and also the retention in the liver of parenterally administered copper was lower than for the other three breeds used in this study. The lower absorption and hepatic storage of copper suggests that this may be associated with the higher incidence of 'steely wool' in Merino fleeces (Marston, 1955), compared to the British breeds grazing the same pastures. Edgar *et al.*, (1941), also found that Merinos were less susceptible than British breeds of sheep to copper toxicoses as they accumulate less copper in the liver.

The original work by Wiener *et al.*, (1969), which was concerned with the absorption of copper from the diet, indicated a difference between breeds in plasma copper concentration, but on intravenous repletion of similar sheep they found no differences between breeds. Herbert *et al.*, (1978), studied the liver retention of copper absorbed from the diet and found breed differences and concluded that the difference was in the absorption of copper from the diet and not as a result of selective grazing.

The breeds of sheep used in the experiments in Europe, especially the North Ronaldsay, Welsh Mountain, and Cheviot in Britain, and the Texel and the White-headed Mutton sheep in Europe, have been in almost complete isolation for many years, and in some

cases centuries, and have adapted to a particular habitat. The North Ronaldsay, for example, has become a very efficient absorber of copper when its principal diet for nine months of the year is seaweed. Herbert *et al.*, (1978), suggest that seaweeds may have a high sulphur content which will inhibit copper absorption, but analysis of twelve varieties of seaweed found on the New Zealand coast showed a copper content of 0.6-0.9 ppm D.M. (Farquharson, unpublished data), which would suggest that the North Ronaldsay may have become very efficient at absorbing copper due to the low copper content of the diet.

The three British breeds of sheep used in this experiment have all been farmed in New Zealand for over 100 years, and have shared the same environment and had access to a similar diet. Therefore it is not surprising that there is no significant difference in their absorption rates of copper from the diet. The Merino breed however has been adapted to a drier environment and in New Zealand is mainly confined to the South Island high country. This is a distinctive environment which may provide sufficient copper to meet dietary requirements for Merinos, so that Merinos do not need to be as efficient in the absorption of copper as other breeds of sheep, which generally graze a different type of pasture.

Although the differences in the absorption of copper between the Merino and the British breeds of sheep may be explained by environmental adaptation, this cannot be used to explain the suspected difference in liver uptake of copper after parenteral copper supplementation. As the Merinos appeared to have accumulated less copper from both the diet and from parenteral copper, it is suggested that two mechanisms could be involved. Either, the Merino has a higher metabolic requirement for copper, or it has a higher excretory rate of copper from the liver, or both mechanisms may operate. Woolliams *et al.*, (1983), suggested that breed variation in copper requirements was the result of the difference in the rate of loss of endogenous copper.

From the results obtained in this experiment, it would appear that there is little merit in changing the breed of sheep, when farming British breeds, to help alleviate potential copper deficiency.

In this experiment insufficient animals were present to establish whether differences in the absorption of copper, between breeds of sheep, did exist, although a difference between the Merino and the British breeds of sheep was suggested. Therefore it would be appropriate to repeat this experiment using greater numbers of animals to confirm these findings. It would also be important to treat similar groups of animals with parenteral copper to determine whether there is any breed difference in the retention of copper in the liver storage cells, in addition to a possible difference in the intestinal absorption of copper.

CONCLUSION

There appears to be some difference in copper pharmacokinetics between the Merino breed of sheep and the British breeds of Border Leicester, N.Z. Romney, and Suffolk. The difference is probably associated with the absorption rate of copper from the diet, as well as the retention of copper in the liver following the administration of copper parenterally.

CHAPTER 7: COPPER CONTENT OF WOOL FROM SHEEP OF KNOWN

COPPER STATUS

INTRODUCTION

Copper is essential for the growth and formation of crimp in the wool fibre (Underwood, 1971). It is required in the keratinization process, when the cross-linking of amino-acid disulphide groups form to produce keratin (Burley & de Kock, 1957; Kapoor et al., 1972). The enzyme required in this process is cytochrome oxidase (Gillespie, 1964).

In copper deficiency there is an associated reduction in wool growth (Underwood, 1971). Such wool has reduced fibre strength, lacks crimp, loses its lustre, and is known as 'steely wool' (Marston, 1955). Copper deficiency also causes a reduction of pigment in black-coloured sheep. This is the result of a reduction in the conversion of the amino-acid tyrosine to melanin; the conversion being catalysed by the copper-containing enzyme, polyphenol oxidase (Underwood, 1971). The two changes of wool seen during a severe dietary deficiency of copper; namely, steely wool and decrease in pigmentation, are reversed within two days of returning to a diet supplying adequate copper (Marston, 1955).

Estimations of copper concentrations of wool from sheep in various parts of the world differ markedly (Table 7.1). Stevenson & Wickham (1976) noted that during the winter the copper content fell as the feed intake was reduced, while Kapoor et al., (1972), found no correlation between dietary copper content and wool copper content. Healy et al., (1964) suggested that wool may be a secondary excretory pathway for copper for those sheep that had adequate dietary copper, and Rish (1970) also supported this theory in relation to zinc and molybdenum. This latter theory suggested that when sheep have low concentrations of stored copper, zinc and molybdenum become stored at higher concentrations in the wool.

Workers	No. of Sheep	Range or mean in ppm D.M.
Cunningham & Hogan (1958)	6	8.3-13.3
Burns <u>et al.</u> (1964)	50	25
Healy <u>et al.</u> (1964)	30	42-147
Healy & Zieleman (1966)	32	22-81
Rish (1970)	n.s.	8-10
Kapoor <u>et al.</u> (1972)	36	3.95-12.55
Stevenson & Wickham (1976)	66	25.5-37.3
Langlands <u>et al.</u> (1981)	12	5.2
Grace (1983)	50	7.0 \pm 0.31
Woolliams <u>et al.</u> (1983)	32	3.48 \pm 0.16
Suttle & McMurray (1983)	5	3.6 \pm 0.35

n.s. = not stated

Table 7.1: Copper content of fleeces determined by other workers.

As many of the experiments carried out in this study were to assess the amount of stored copper in the sheep, it seemed appropriate in the current set of experiments to determine the copper concentration of the wool. The investigation was attractive too because wool is a convenient tissue to collect and might possibly indicate the copper status of the animal at the time of keratinization of the wool fibre.

MATERIALS AND METHODS

Reference has been made previously to the collection of wool samples from certain sheep during the process of the other experiments in this study. In summary, samples were collected from the two pilot sheep and the nine other sheep used in setting up the

toxicity model (Chapter 2). The sheep in the experiment comparing copper uptake and retention between different breeds of sheep were used (Chapter 6), and also those sheep involved in monitoring the copper deficient and the copper sufficient farms (Chapter 5).

The samples of wool were collected by close-clipping over the site of entry for the liver biopsy (the right paralumbar fossa). In the sheep which were subjected to the taking of repeated liver biopsy samples, only that wool that had grown since the previous occasion was collected.

Each wool sample was washed at least three times in a detergent¹, to remove the wool grease and any extraneous matter. The sample was then washed a further three times in deionized water, dried in the oven at 100°C for 72 hours, cooled to room temperature in a dessicator and weighed.

Each weighed sample of approximately 300mg was digested in concentrated nitric acid and taken to dryness. The digest was then taken up in 5ml of 2N hydrochloric acid ready for copper estimation using the atomic emission spectrometer (Appendix 4).

The estimates of the copper content of the wool for each sheep were compared against both the liver copper concentrations of that sheep, and blood copper concentrations of samples collected on that same day.

The linear correlation coefficient was computed using the Pearson product moment correlation coefficient.

¹Pyroneg, Diversey Wallace Ltd., New Zealand

RESULTS

The values for copper concentrations from the analysis of the 168 wool samples ranged from 1.10 - 11.56 ppm Cu D.M., with a mean of 4.75 ± 0.14 ppm (Figure 7.1). Although the correlation between copper content of wool and the copper content of liver was low ($r = 0.412$); of the 39 liver copper values below 50 ppm Cu D.M. only two sheep had wool values in excess of the mean wool value of 4.75 ppm.

The histograms of the liver copper concentration and the blood copper concentration are shown in Figures 7.2 and 7.3. The correlation between wool copper concentration and blood copper concentration from the same sheep was low ($r = 0.061$).

Even in those sheep that received weekly injections of 50mg of copper, there was only a very small increase in the copper content of the wool. Of the 63 sheep with a liver copper concentration in excess of 500ppm Cu D.M., fifty of these animals had wool containing copper in excess of the mean of 4.75ppm. The low correlation between wool copper concentration and liver copper concentration was most evident in animals of lower liver copper concentration, but as liver concentrations increased so did the wool copper concentration; the regression line for this relationship had a slope of

$$y = 4.19 + 0.000874 x.$$

DISCUSSION

The copper content of wool samples collected during this series of experiments was generally lower than those amounts obtained by most of the workers listed in Table 7.1. This is significant especially as many of the sheep in the current study had high liver copper concentrations. Rish (1970) and Healy et al. (1964) suggested that wool may be a secondary excretory pathway for copper. On the basis of the results obtained in this series of experiments this premise must be doubted, as although 50 out of the 63 sheep with

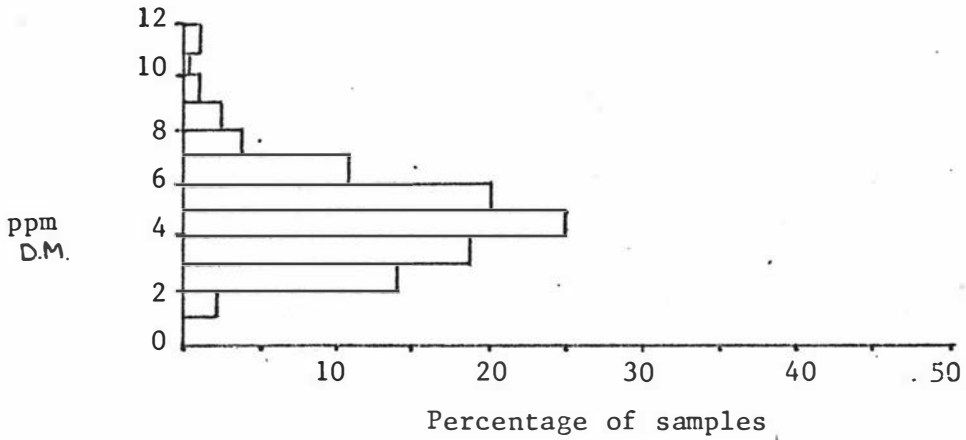


Figure 7.1: Copper concentration of wool samples

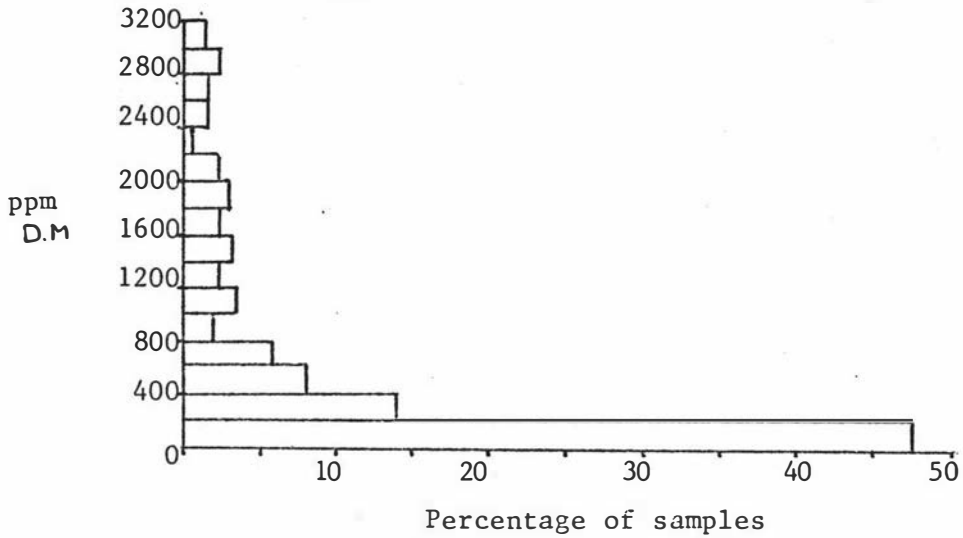


Figure 7.2: Copper concentration of liver tissues

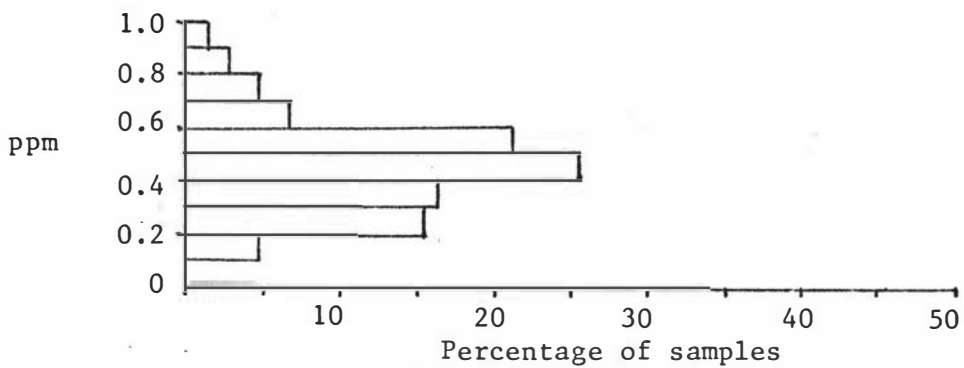


Figure 7.3: Copper concentration of blood samples

liver copper concentrations in excess of 500 ppm had copper concentrations in wool greater than 4.75 ppm, the highest copper concentration found in any wool sample was only 11.6 ppm.

Similarly the results of Burns et al. (1964), Healy & Zielemann (1966), and Stevenson & Wickham (1976) must be treated with reserve. For a sheep to deposit in excess of 20ppm of copper into a 3kg clean fleece annually would require at least 2.7mg Cu/day in the diet (assuming an absorption of 6%) just to support wool growth. This is almost 50% of the estimated copper requirements for body maintenance (Grace, 1983). The results of analyses of wool samples performed since 1980 gave the lowest values for copper content (Table 7.1). Some of the earlier workers used unwashed samples of wool and as the fleece may collect foreign material, variable results may have occurred.

Normal, soft, animal tissues (Grace, 1983), with the exception of liver, contain a mean copper content of 4.2 ppm D.M. (Underwood, 1971). It is suggested that wool utilises copper similarly to a normal soft tissue and has a normal copper requirement, and thus the mean copper content obtained in this study of 4.75 ppm D.M. appears to fall in the expected range (Grace, 1983).

Woolliams et al., (1983), using unwashed wool samples found a correlation between wool and liver copper concentration ($r = 0.46$), which agrees closely with the correlation of 0.412 obtained in this study. They also found that copper concentrations in wool rarely exceeded 4.5 ppm, but that the concentration did continue to rise slightly in sheep having liver copper levels in excess of 20 ppm. A similar relationship occurred in this study.

The analysis of wool for copper content appears to have little merit in indicating the copper status of animals which have adequate or high copper reserves. When the liver concentrations were below 20 ppm, Kellaway et al. (1978) found that the copper content of both bovine hair and bovine plasma were sensitive to changes. Suttle &

McMurray (1983), in their repletion experiments found that the copper status of wool changed following copper depletion and with subsequent repletion of the animal. They concluded that wool was a good indicator of the copper status of the animal as wool copper concentration changed less rapidly than either liver or plasma copper concentration. Langlands et al., (1981), when investigating the relationship between wool copper content and hepatic copper storage found that wool copper content appeared to be a reliable indicator of induced copper deficiency. Insufficient numbers of sheep of low copper status were used in these Massey experiments to ascertain the value of wool analysis in detecting copper deficiency.

CONCLUSION

The analyses of 168 wool samples from sheep of known liver copper status are presented. The wool samples had a mean copper concentration of 4.75 ± 0.14 ppm. Sheep of high copper status do not accumulate copper in the wool fibres. An insufficient number of animals of low copper status were used in these experiments to indicate the value of wool in the diagnosis of copper deficiency.

GENERAL DISCUSSION

The work described in this thesis covers two aspects of copper metabolism. First the factors that may enhance the toxicity of copper administered therapeutically are considered, and secondly observations have been made on the changes in copper status of animals grazing on New Zealand pastures. As pasture is the stable diet of sheep in New Zealand, it was preferable for the sheep used in these experiments to be grazed on pasture whenever possible. This is in contrast to studies in other countries where many of the experiments involved animals which were housed and fed concentrate diets (Dick, 1954 ; Wiener, 1980; Suttle, 1981b). Similarly in the earlier work in New Zealand of Hogan *et al.*, (1968), pen-fed sheep were used. As trace element concentrations in pasture change seasonally and also vary under other circumstances, it was anticipated and ultimately shown that some of the copper concentrations recorded in sheep proved to be different from those of previous workers. These differences were largely attributable to the effects of pasture feeding.

In both the area of toxicity and seasonal copper status of animals, new information has been obtained, and where possible, explanations have been offered for the variations reported. Further, these variations open up challenging new areas for investigation which need to be undertaken before a more complete understanding of copper supplementation to livestock in New Zealand is available.

In addition to confirming the results of previous studies on the development of copper toxicity, namely that serum concentrations of sorbitol dehydrogenase and serum glutamate oxaloacetate transaminase become elevated, and that liver copper concentrations increase; the present study highlighted responses to the repeated parenteral administration of copper. It would appear that sorbitol dehydrogenase is the earlier indicator of hepatic cellular damage as

serum concentrations of this enzyme are elevated from one to four weeks before any significant rise in serum concentrations of serum glutamate oxaloacetate transaminase activity can be detected. The latter is the enzyme that has been measured most often by previous workers (Table 1.4). There was a regular increase in both the number of hepatocytes damaged and in the accumulation of copper granules as the number of copper treatments increased, until a haemolytic crisis or death occurred. At this stage approximately 50% of the hepatocytes were destroyed. These histological changes did not appear to be related to the number of treatments administered, nor to the copper concentration of the liver at the time of that injection. The amount of copper present in the hepatocytes before any change in the serum enzyme concentrations was observed, and before there were alterations in the cellular structure, varied between sheep. This suggests that there is an individual threshold of tolerance to copper for each sheep and the mechanism may be either an inherent ability of the hepatocytic lysosomes to retain the accumulated excess copper, or some undefined resistance factor operating elsewhere.

The analysis of liver tissue for copper content at the time of death or during the haemolytic crisis showed that the copper concentration was approximately half of that recorded at the previous sampling. This suggests that either half of the hepatocytes are destroyed and release their copper contents, during a crisis, or there is a massive release of lysosomal copper from the intact hepatocytes. Alternatively both phenomena could have taken place.

In those sheep that reduced their feed intake for two or three days and then died, from causes other than copper toxicity, there was also a reduction of almost 50% in their liver copper concentration at the time of death. This reduction may be important when field assessments of the copper status of grazing animals are being undertaken. Not infrequently, veterinarians have submitted samples of liver for copper analysis from animals which are already suffering from other diseases and are in poor body condition. The results from the current work show that in the determination of copper

concentrations in grazing animals, the selection of those individuals which are free of concurrent disease and in good body condition is very important. Accordingly it should be emphasised that the liver samples collected for copper estimation should be taken either by liver biopsy, or from healthy animals destroyed for that purpose. The liver biopsy technique has been performed regularly in this series of experiments and has proved both reliable and safe.

The individual variations in liver copper content may also pose difficulties in the diagnosis of copper toxicity. It would appear that some sheep can tolerate in excess of 4,000 ppm D.M. of copper in the liver, without showing any untoward effects, while in others, with concentrations as low as 1400 ppm D.M. of copper, clear signs of copper toxicity are present. Therefore the diagnosis of copper poisoning is dependent on a consideration of the clinical signs confirmed by the presence of elevated liver copper. It should be noted also that during the haemolytic crisis, the kidneys accumulate large amounts of copper, the measurement of which is a better confirmatory criterion for copper poisoning than the liver estimation. To confirm that death has resulted from copper poisoning the history, clinical signs and autopsy findings should be considered in relation to the histopathological changes in the liver and the kidney, and the copper concentrations measured in particularly the kidney, as well as the liver.

Work from this thesis suggests that the analysis of wool samples is not a suitable method for estimating the copper status of sheep which are absorbing copper in excess of their requirements.

In previous reports (Skinner, 1960; Ross, 1964) it has been stated that copper poisoning has been initiated by stress following the use of copper therapeutics. On many New Zealand farms sheep are placed under temporary stress as a result of routine management procedures, and it is often during these periods that sheep are dosed with copper. A series of experiments was designed in an attempt to identify those factors which may potentiate the toxicity of

parenterally administered copper. The effects of anthelmintic administration, insecticide application, pregnancy, dehydration and exposure to a cold environment appeared to have no potentiating effect. However some factors did enhance the toxic effects of copper preparations given parenterally. These included starvation, exposure to a hot environment, and the harbouring of a heavy burden of gastrointestinal parasites. These latter three conditions probably affected sheep in a similar manner; that is, by a reduction in the intake of nutrients. Also in those sheep affected with gastrointestinal parasitism there was a greater susceptibility to copper toxicity as these animals were of a younger age. A similar finding was recorded by Lewis et al., (1981).

In this study it was shown that when sheep are fasted for 24 hours or more there is an alteration in the plasma protein concentration, and after the administration of copper, the blood copper concentration became significantly elevated. A most likely explanation for this phenomenon may be as follows. During fasting the body maintains gluconeogenesis by utilising amino-acids. These immediately available amino-acids are held as a pool in the hepatic lysosomes (Aronson, 1980). It would appear that because of this demand for amino-acids by the fasting animal, autophagy of the lysosomes occurs, which may also cause the release of their accumulated copper, or render hepatocytes unable to take up circulating albumin-complexed copper for storage, or both mechanisms may be involved. At the same time there is an increase in plasma albumin, which is presumably released from the hepatocytes, which will transport the copper ions from the site of injection. Therefore it would seem that to minimise the risk of poisoning following parenteral administration of copper, animals should be in good body condition and not starved as a result of prolonged periods of yarding or droving without food.

Young animals are more susceptible to copper poisoning than adults. Therefore to reduce the risk of death following dosing, care must be given to the dose rate used. Alternatively the foetus can

gain a store of copper in utero by dosing the dam at an appropriate stage of pregnancy. This will ensure an adequate supply of copper to the foetus during pregnancy and subsequently during lactation.

The measurement of the blood copper concentration at hourly intervals after the administration of parenteral copper appeared to be an effective technique for measuring the rate of uptake, and therefore the translocation rate, of copper formulations given under various controlled conditions. It also indicated the potential toxicity of copper compounds when their translocation rate was compared to the translocation rate of safe formulations of known uptake rate. The measurement of the activity of sorbitol dehydrogenase and glutamate oxaloacetate transaminase enzymes in the serum after 48 and 96 hours, indicated the amount of cellular damage to hepatocytes caused by parenteral copper formulations. By measuring the rate of uptake of a copper compound and by measuring the activity of the liver enzymes in serum, it should be possible to determine the potential toxicity of that copper compound in any particular formulation. It should also be possible to determine which stressors may enhance the toxicity of copper.

In this study starvation was the stressor found most likely to enhance copper toxicity, and it would be appropriate now for further biochemical studies to be undertaken to investigate more precisely the changes that occur in liver metabolism in response to starvation. The understanding of this mechanism is important as many therapeutic agents are initially stored in the liver, and their ultimate fate is influenced by variations in the metabolism of the liver.

The determination of the copper status of animals grazed on pasture, and the estimation of the copper content of the plants that make up that pasture, have not been regularly recorded. Most experimental investigations have used animals in a controlled environment and on a diet of known composition. In this study the regular monitoring of animals and pastures on New Zealand farms has highlighted the dynamic nature of copper metabolism. It has also

identified factors that may influence the absorption of copper from pasture and its retention in the liver.

This study has also shown the poor correlation which exists between the copper content of the soil and the copper content of the pasture growing on that soil. Like the uptake of copper from pasture by animals, the uptake of copper from soil by plants is influenced by a variety of factors (Le Riche et al., 1963; Mitchell, 1974).

Although there are variations in pasture copper concentrations between farms, these are not as great as the variation in liver copper concentrations found in the animals grazing those pastures. As outlined in this thesis, and as reported in previous work, pasture copper concentrations usually bear little relationship to liver storage concentrations in sheep. The factors that appear to influence the absorption and storage of copper are the inhibiting elements of molybdenum and sulphur incorporated in the plant, and the soil contaminating elements of zinc, iron and manganese. Other important factors which influence copper uptake are the species of plant ingested, the digestibility of that plant and its stage of maturity, and the copper status of the sheep grazing that pasture.

The elements that inhibit copper absorption, namely molybdenum and sulphur, have been well investigated in sheep on hand-fed diets, and their effects quantified (Suttle & Field, 1968b). However the effect of these two elements does not completely explain the changing copper status of animals grazing pasture. It has been noted again in this study that the soil elements of zinc, iron and manganese may affect copper uptake and storage. These elements contaminate pasture during winter in particular, and therefore may have a marked influence on copper absorption and retention (Field & Purves, 1964; Ghergariu, 1978). Further, winter is the time of the year when pasture copper content is at its lowest. Reynolds (1979) and Standish et al., (1971) have reported on the influence of zinc and iron respectively. In this study and from the analysis of pasture on other New Zealand farms, it has been noted that when pasture

manganese concentrations rise above 200 ppm D.M. that sheep grazing such pasture frequently have low liver copper concentrations. On the other hand sheep grazing pasture with a lower manganese content (> 100 ppm D.M.) usually show an adequate liver storage of copper. These observations are worthy of further study particularly as manganese is stoichiometrically similar to copper in the ionic state and may be able to replace some copper ions in their protein complex without those proteins losing any of their activity. The role of manganese as a possible antagonist to copper requires further investigation. On investigation of the farms where the animals had adequate or high copper storage, it was established that the predominant species of plant in the pasture were perennial ryegrass (Lolium perenne) and white clover (Trifolium repens). This contrasted with the farms on which the concentrations of liver copper in the sheep were low. On these farms the predominant pasture species were the less digestible grasses such as crested dogstail (Cynosurus cristatus), browntop (Agrostis tenuis), and danthonia (Sieglingia decumbens). Probably it was not so much a marked variation in the copper content of the species in these pastures per se, but their differing digestibility which affected copper uptake. The digestibility of plant material is influenced by the species and also by the stage of maturity (Church, 1977).

The liver copper concentrations of sheep showed not only a variation between farms, but also a seasonal variation. The copper content of the pasture was lowest in the winter and early spring on all farms studied. During this same period, pasture molybdenum concentrations were highest and the soil contaminating elements of iron, zinc and manganese were at their highest in the pasture. This seasonal variation was most marked on farm A (the copper deficient farm described in Chapter 5) where the sheep were set-stocked on the poorer pastures during the winter. As the winter progressed these sheep would only have had access to the less digestible pasture species, and then at a time when requirements for copper were highest.

In this work the measurement of the amount of copper retained in the liver was used in calculating the absorption of copper from the diet. In sheep grazing pasture, the amount of copper retained in the liver varied from 4.7% to 14.3% of dietary copper. Although the pasture content of inhibiting elements, and the species of plants that comprise that pasture influence the absorption rate, it would appear from this work that the digestibility of the plant species, and the copper status of the animals grazing that pasture have a dominant influence on the absorption of copper from the diet. The farm on which the sheep retained 14.3% of dietary copper had pastures containing mainly perennial ryegrass and white clover; both in an active growing state. The contrast in copper retention between the "copper deficient" and the "copper sufficient" farms is the most surprising. Those sheep grazing the "copper deficient" farm and being of low copper status retained 6.4% of the dietary copper, whereas those sheep grazing the "copper sufficient" farm and maintaining their copper status retained only 4.7% of the dietary copper. These results are similar to those of Kirchgessner et al., (1981) which showed that the excretory and absorptive mechanisms for copper tend to adapt to the variations in copper requirements and the copper status of the animal, thus altering the absorption rate of copper from the diet.

The sheep that were fed on the synthetic diet of very small particle size, retained 76.6% of the dietary copper in the liver. This high retention rate is comparable to that of the pre-ruminant lamb (Suttle, 1979) and suggests that in the digestion of this diet it almost completely by-passed the rumen, which is the major site of interference of copper absorption (Suttle, 1981c).

Suttle, (1981a), has estimated the absorption of copper from various diets (Table 1.2), and has also shown the effect of molybdenum and sulphur content in the diet on the amount of dietary copper available to the animal. In New Zealand where sheep are grazed continuously on pasture of varying digestibility and copper content, the amount of copper available to the animal from the diet

is extremely variable.

When investigating farms to establish their copper status, it is imperative that plant and animal tissue copper concentrations should be evaluated at regular intervals. From this data and from information on the availability of copper in various plant species, it should be possible to determine the copper requirements of the animals relative to the available copper of the pasture. If the pasture is unable to supply sufficient copper to meet metabolic requirements, then copper supplementation should be provided. Alternatively it may be possible to supply adequate copper from pasture by changing the grazing management of that farm.

From the data collected on the "copper deficient" farm and on the "copper sufficient" farm, it appears that 50 mg of parenteral copper given to ewes in early winter, is sufficient to maintain adequate blood and liver copper concentrations during pregnancy. In sheep with adequate copper reserves at the outset, it appears that liver copper concentrations usually increase after treatment and remain at this higher level.

On any farm where copper supplementation is practised the copper status of the farm and of its animals should be assessed at least annually, prior to medication. If sheep of adequate copper status are maintained on a diet providing sufficient copper to meet metabolic requirements and are also given supplementary copper, toxicity may result. The supplementary copper will accumulate in the liver and may reach the threshold level for copper storage and precipitate copper toxicity. This accumulation of copper sufficient to reach the threshold level to precipitate toxicity may involve more than one treatment.

The variation in the absorption of copper from the diet and the retention in the liver between the British breeds of sheep (the Border Leicester, the Suffolk, and the New Zealand Romney), used in this study, was non-significant. Therefore manipulating the breed of

sheep to graze pastures of a lower copper content appears to be of little value. The Merinos used in this experiment appeared to have a higher turnover of copper and therefore a higher requirement for copper, as was found by Edgar *et al.*, (1941). However in New Zealand the British breeds of sheep are not adapted to the environment grazed by Merinos and so a change of breed to counter copper deficiency would be impractical.

The copper requirements of sheep of different breeds have been shown to be variable in experiments carried out in Britain (Wiener, 1980) and in Europe (van der Berg, 1983). However, the breeds of sheep used in these European experiments have adapted to their respective environments over many years, whereas the British breeds of sheep used in this study have shared a common environment. This experiment should be repeated using a greater number of animals from a greater range of breeds, in an endeavour to establish the variability in copper absorption that exists in the breeds of sheep farmed in New Zealand. Similar experiments using cattle of different breeds, and also of deer, would provide valuable information for farming in this country.

The information gained from this thesis suggests that copper toxicity resulting from the treatment of sheep with copper therapeutic agents can be avoided. Before initiating copper therapy for sheep, the copper status of the animals and the pasture on which they are grazing must be established. It is suggested that in New Zealand, the pasture should provide sufficient copper to meet the daily requirements of the animals grazing that pasture, and that the liver copper concentration should be in excess of 50 ppm Cu D.M. If these criteria can not be met then copper supplementation must be considered.

Further, the therapeutic copper formulation used must have an adequate margin of safety. This can be determined by using copper compounds of a known translocation rate that will not produce damage to hepatic cells. The formulation of the copper therapeutic agent

should not be altered until it is established that this will not increase the translocation rate of the copper. Any suitable preparation must be administered at the appropriate dose rate based on the age and on the bodyweight of the animals being treated.

In addition, any stressors that may potentiate the toxicity of copper by increasing its translocation rate should be avoided. The stressors known to enhance toxicity are those that reduce feed intake, which can be the result of starvation, exposure to a hot environment, or when animals are affected with gastrointestinal parasites.

While copper given parenterally is the most reliable and the most commonly used form of copper therapy, its use can result in toxicity. It is important that in prescribing and using these products that their potential toxicity is recognised but as this toxicity can be avoided, the pre-requisites for safe copper medication should be widely promoted. Although study should continue to establish those factors which may enhance copper toxicity, it is equally important to develop copper therapeutics that are safer than existing products.. Such work may lead to different formulations, different routes of administration, or the development of slowly absorbed formulations in controlled release devices.

APPENDICESAPPENDIX ITechnique for the Analysis of Blood Samples

The following steps were used:

1. One ml of blood was placed in a test tube
2. Half one ml of 25 volume hydrogen peroxide was added and left for 4 hours
3. Two ml of concentrated nitric acid was added and the solution was taken to dryness
4. The residue was dissolved in 5 ml of 2N hydrochloric acid, containing 5 ppm Ni to act as an internal standard, and warmed for 45 minutes
5. A blank, containing deionised water instead of blood, was run with each batch of samples
6. The solution was analysed for mineral content using the inductively-coupled argon plasma emission spectrometer

All glassware was washed, soaked in Pyroneg detergent (Diversey Wallace Ltd) for 12 hours, soaked in 2N hydrochloric acid for 8 hours and then triple rinsed in deionised water. They were air-dried.

APPENDIX IITechnique for the Analysis of Tissues and Pastures

The following steps were used:

1. A sample of tissue was placed in a weighed crucible and dried at 100°C for 15 hours
2. The sample and crucible were weighed
3. The sample was ashed at 485°C for 15 hours
4. The ash was washed into a test-tube with approximately 5 ml of 2N hydrochloric acid and taken to dryness
5. Two ml of concentrated nitric acid was added and taken to dryness
6. The residue was dissolved in 5 ml of 2N hydrochloric acid and warmed for 45 minutes
7. A blank sample was prepared using every step except the sample
8. The solution was analysed for mineral content using the inductively-coupled argon plasma emission spectrometer
9. Pasture samples were dried at 100°C for 15 hours and milled to 2 mm. Approximately 0.5 gm of sample was weighed into a crucible and ashed at 485°C for 15 hours. The ash was then digested as the tissue samples.
10. Pasture samples used to estimate sulphur content were wet-ashed in 2 ml of concentrated nitric acid instead of dry-ashed at 485°C.

11. In every batch of samples analysed a standard of known mineral content was used. This was the National Bureau of Standards Bovine Liver Standard Reference Material No. 1577 which contained 193 ± 10 ppm of copper

12. All glassware and crucibles were prepared as set out in Appendix I.

APPENDIX IIITechnique for Demonstrating Copper in Tissue Sections

Staining solution

- a. 50 mg rubeanic acid (BDH Ltd) in 50 ml ethanol
- b. 10 gm sodium acetate in 100 mls distilled water staining solution

Use 2.5 ml of solution a and 50 ml of solution b.

Method:

1. Sections of fixed tissues were placed into distilled water
2. Sections were placed in covered Coplin jars containing stain, at 37°C, overnight
3. The sections were rinsed in 70% ethanol for 2 to 3 minutes
4. The sections were then rinsed in ethanol coloured with eosin, until the sections were light pink
5. Each section was cleared in xylol and mounted in DPX.

Note: To get consistent results fresh solution should be used.

This technique was supplied by Mr B.J. Young, Ruakura Animal Health Laboratory.

APPENDIX IVTechnique for Analysis of Wool Samples

The following steps were used:

1. A sample of approximately 2 gm of wool was triple washed in hot detergent (Pyroneg, Diversey Wallace Ltd) and triple rinsed in deionised water

2. Each sample was dried at 100°C for 3 days, and cooled in a dessicator

3. Approximately 0.5 gm of wool was weighed into a dried weighed test-tube

4. Two ml of concentrated nitric acid was added and the mixture was taken to dryness. This step was repeated up to four times until the digestion was completed

5. The residue was dissolved in 5 ml of 2N hydrochloric acid and warmed for 45 minutes

6. A blank solution was prepared using every step except the sample

7. The solution was analysed for mineral content using the inductively-coupled argon plasma emission spectrometer

8. All glassware was prepared as set out in Appendix I.

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