Open Research Online

The Open University's repository of research publications
and other research outputs

The effect of molybdenum, iron and sulphur on copper metabolism and physiology of sheep

Thesis

oro.open.ac.uk

THE EFFECTS OF MOLYBDENUM, IRON AND SULPHUR ON

COPPER METABOLISM AND PHYSIOLOGY OF SHEEP

BY

CLAIRE LOUISE WILLIAMS

BSc. (HONS) ANIMAL SCIENCE, UNIVERSITY OF LEEDS

MSc. APPLIED ANIMAL BEHAVIOUR AND ANIMAL WELFARE, UNIVERSITY

OF EDINBURGH

THESIS SUBMITTED TO THE OPEN UNIVERSITY FOR THE AWARD OF THE

DEGREE OF DOCTOR OF PHILOSOPHY

MARCH 2004

ASRC, HARPER ADAMS UNIVERSITY COLLEGE, EDGMOND, NEWPORT,

SHROPSHIRE, TF10 8NB, UK

ProQuest Number: C819055

ProQuest C819055

ABSTRACT

Four experiments were carried out to investigate the effects of the dietary antagonists molybdenum (Mo) or iron (Fe) on copper (Cu) metabolism and physiology of sheep. In experiment 1, dietary Mo or Fe was provided in diets of Charollais cross growing lambs to investigate the effects on Cu status and performance. Dietary Mo or Fe had no effect on lamb performance but there were significant effects on Cu status. Plasma copper (Pl-Cu) concentrations were significantly higher in lambs that received dietary Mo ($P<0.001$) and ceruloplasmin (CP) activities were significantly lower ($P<0.01$) in lambs that received dietary Mo. Dietary Mo significantly altered the CP:Pl-Cu ratio in addition to significantly reducing liver Cu concentrations. No clinical symptoms were observed. In experiment 2, the effect of dietary Mo or Fe on immune responses of Charollais cross growing lambs were investigated. Cell mediated and humoral immunity were not affected by dietary treatment despite liver Cu concentrations being significantly lower ($P<0.001$) in lambs fed dietary Mo. In experiment 3, dietary Mo or Fe was provided in diets to Charollais cross growing lambs to investigate the effects on Cu status and trace element accumulation in the pituitary, ovary, cerebellum and liver. In addition, effects on pituitary function and ovary morphology were investigated. Dietary Mo significantly ($P<0.001$) reduced Cu concentrations in the liver in addition to significantly increasing ($P<0.001$) Mo concentration in the liver and ovary. Dietary Mo was found to significantly increase retention of ACTH in the pituitary gland. There were no conclusive effects of dietary treatment on pituitary or ovary histology. In experiment 4, dietary Mo or Fe was provided in the diets of Scottish Blackface wethers to determine the effects on Cu status and hepatic CP mRNA expression. CP mRNA expression was found to be unaffected by dietary treatment. These studies confirmed that dietary Mo was a more potent antagonist on Cu metabolism and physiology in sheep than dietary Fe.

TABLE OF CONTENTS

Chapter 5 **The effect of dietary molybdenum or iron on copper status, pituitary gland function, ovary morphology and trace element accumulation in ovary, pituitary, cerebellum and liver of growing lambs**

Chapter 6   The effect of molybdenum and iron on copper status and ceruloplasmin expression in the liver of growing lambs

LIST OF TABLES

TABLE OF FIGURES

Part of the work in this thesis has appeared previously:

Williams, C.L., Haywood, S., Loughran, M., Wilkinson, R.G. Illingworth, D.V. and Mackenzie, A.M. 2003 The effect of dietary molybdenum or iron on copper status and trace element accumulation in the pituitary and ovary of growing lambs. In: *Proceedings of the British Society of Animal Science (Annual Meeting), York*

Williams, C.L., Haywood, S., Loughran, M., Wilkinson, R.G. and Mackenzie, A.M. 2002 The effect of dietary molybdenum or iron on copper status and pituitary gland trace element content of growing lambs. In: *3rd International Meeting on Copper Homeostasis and its Disorders: Molecular and Cellular Aspects, Ischia, Italy*

Williams, C.L., Wilkinson, R.G. and Mackenzie, A.M. 2002 The effect of a molybdenum or iron induced copper deficiency on cellular and humoral immune responses in growing lambs. In: *11th International Symposium on Trace Elements in Man and Animals, San Fransisco*

Williams, C.L., Wilkinson, R.G. and Mackenzie, A.M. 2002 The effect of molybdenum or iron on copper status of growing lambs. In: *11th International Symposium on Trace Elements in Man and Animals, San Fransisco*

Williams, C.L., Mackenzie, A.M. and Wilkinson, R.G. 2002 The effect of molybdenum, iron and sulphur supplementation on immune function in growing lambs. In: *Proceedings of the British Society of Animal Science (Annual Meeting), York*

Williams, C.L., Mackenzie, A.M., Illingworth, D.V. and Wilkinson, R.G. 2001 The effect of molybdenum, iron and sulphur supplementation on growth rate and copper status of lambs. In: *Proceedings of the British Society of Animal Science (Annual Meeting), Scarborough*

ACKNOWLEDGEMENTS

Author declaration

I declare that this thesis has been composed entirely by myself and that it has not been accepted in any previous application for a degree. The work, of which it is a record, has been done by myself. Quotations have been distinguished by quotation marks and sources of information have been specifically acknowledged.

Claire Louise Williams

Literature Review

1.1. Introduction

At least twenty-two mineral elements are believed to be 'essential' for higher forms of animal life (Underwood, 1977). Of the essential elements, all have been shown to have one or more metabolic functions and have been classified as being 'essential' to maintain normal cellular metabolism (Spears, 1999; Lee et al., 2002). An essential mineral element has previously been defined as:

"one that is required to support adequate growth, reproduction and health throughout the life cycle when all other nutrients are optimal"

(O'Dell and Sunde, 1997)

The essential mineral elements are classified as either major or trace elements depending upon their concentration in the animal or the amounts required in the diet. Major elements are expressed in g kg^{-1} and trace elements are expressed in mg kg^{-1}. The major elements include calcium (Ca), phosphorus (P), potassium (K), sodium (Na), chlorine (Cl), sulphur (S) and magnesium (Mg) and the trace elements include iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo), selenium (Se), manganese (Mn), cobalt (Co) and iodine (I) (Judson and McFarlane, 1998). Elements including arsenic (As), nickel (Ni), silicon (Si) and vanadium (V) may also be required in the diet, but no specific biochemical function has yet been identified for them (Judson and McFarlane, 1998).

Inadequate and excessive intakes of mineral elements may lead to deficiency or toxicity respectively. For example, inadequate intakes of Cu may induce a primary Cu deficiency. Additionally, a mineral element may be metabolically unavailable due to other antagonistic dietary components that interact with the element and reduce its' availability (Suttle, 1991). A secondary Cu deficiency may arise due to the complex antagonistic interactions

1

of dietary Mo and S (Suttle, 1975). In addition, dietary Fe and S are known to affect Cu metabolism (Suttle *et al.,* 1984; Bremner *et al.,* 1987). This review will discuss the nutritional, physiological and biochemical role of Cu and its' interactions between Mo and/or S and Fe and S in ruminant animals.

1.2. Properties of copper

1.2.1. Physical and chemical properties of copper

Copper is a d-block transition element with the atomic number of 29 and an atomic weight of 63.546 daltons. It occurs in the environment as one of three oxidation states: Cu^0 (copper metal), Cu^{1+} (cuprous ion) or Cu^{2+} (cupric ion) (Georgievskii *et al.,* 1982). The cuprous state (+1) is easily oxidised to the higher oxidation state and is stable only in very insoluble compounds. Therefore, in most compounds, Cu is present as Cu^{2+} (Linder, 1991).

1.2.2. Dietary Sources

Copper is a natural element and is widely distributed in feedstuffs as illustrated in Table 1.1. Sources rich in Cu are whole grains, nuts, legumes, seeds, seed by-products and shellfish. Grasses tend to have a lower Cu contents than legumes, and grains tend to be higher than leaves or stems (Hartmans and Bosman, 1970; McDowell, 1992).

Table 1.1. *Copper contents of animal feedstuffs*

Dietary source	Copper content (mg kg^{-1})
Barley grain	5-9
Barley straw	3-5
Brewers grains	21
Citrus pulp	5-7
Fresh grass	7-8
Grass silage (early)	11
Grass silage (late)	3
Grass hay (good quality)	9
Maize	2.5
Rape seed	5-7
Soyabean meal	25
Sugar beet pulp (molassed and dried)	11-13
Turnips	21
Wheat grain	5-7
Wheat straw	4

Sources: McDowell (1992), McDonald *et al.* (1995) and NRC (1985)

Suttle (1983a) found that Cu is well absorbed from foodstuffs low in fibre such as cereals (A_{Cu} 9.1 %) but poorly absorbed from fresh herbage including grass (A_{Cu} 1.4 - 2.5 %). Conservation of grass as hay or silage generally improves Cu availability (Suttle, 1983a). In most circumstances, Cu concentrations decline as plants mature, and are lower from alkaline soils (McDowell, 1985). The most influential factor affecting Cu metabolism from feedstuffs in ruminants is the presence of Mo in association with S (see section 1.2.3) (Lee and Grace, 1997).

1.2.3. Soil and herbage influences on copper metabolism

The Cu content of crops can be related to geographical location, soil Cu levels (Beeson *et al.*, 1947), plant species, stage of maturity, yield, seasonal and temporal climatic factors, soil pH, fertiliser application and soil fertility (McFarlane *et al.*, 1990; MacPherson, 2000). Contamination of the soil due to Cu toxicity may arise from sewage sludge, deposition of industrial wastes and industrial pollution (Pais and Jones, 1997). Although many soil types in the UK have adequate Cu contents (normal range 2 – 60 mg kg^{-1})(Thornton, 1983), liming or reseeding to 'improve' growth of upland pastures causes a reduction in availability of Cu in soils due to a rise in pH. Therefore, Cu uptake from plants is pH dependent (McFarlane *et al.*, 1990). The increase in alkalinity of the soil caused by the application of lime to pastures may result in increased uptake of Mo by pasture plants (Mengal and Kirkby, 1987). An increase in liming of lakes, wetlands and pastures to counteract the effects of acid rain in south-west Sweden, has been attributed to an increase in a secondary Cu deficiency-type disease in moose, observed since the mid 1980's (Frank, 1998; Frank *et al.*, 2000a, 2000b).

Molybdenum availability is increased by liming, with Mo concentrations in soils averaging between 1-2 mg kg^{-1} (Cannon *et al.*, 1978). Acidic soils are often deficient in Mo whilst alkaline shale and other sedimentary related soils contain high concentrations of Mo (Mengal and Kirkby, 1987). Humus rich peat soils contain high levels of Mo such as the

'teart' pastures of Somerset (Ferguson *et al.*, 1943). Ferguson *et al.* (1943) first noted clinical symptoms attributable to a secondary Cu deficiency by an increased incidence of scouring and change in coat colour of Red Devon cattle when put out to graze on the 'teart pastures' containing between 20-100 mg kg^{-1} Mo (see section 1.6.1).

Animals ingest soil as they graze (Suttle *et al.*, 1975) and it has been estimated that cattle and sheep may ingest between 100-300 g soil kg^{-1} herbage DM respectively on short pastures (Dewes, 1996). Soils known to contain low levels of Cu include leached sandy soils, calcerous sands and soils with low organic matter contents. Soil ingestion may increase intakes of Mo or Fe subsequently altering Cu metabolism (Suttle *et al.*, 1975). Soil ingestion may also increase in areas of poor drainage or where pasture growth is poor. Also, soil ingestion may increase when feeding root crops covered in mud or dust or in areas with high stocking densities (Dewes, 1996).

Iron contents of soils vary considerably with pasture contents ranging from 250-400 mg kg^{-1} Fe, with very high levels indicative of soil contamination (>6000 mg kg^{-1}) (Judson and McFarlane, 1998). High dietary intakes of Fe may be due to soil consumed directly, in feedstuffs or from mineral supplements containing iron oxides. Increased intakes of Fe may occur from grazing short pastures resulting in an increase in soil intake, soil contamination during ensiling or during fodder crop harvesting. In New Zealand, pasture Fe concentrations have been categorised according to the level of soil contamination: severe (>6000 mg kg^{-1} Fe DM), high (3000-6000 mg kg^{-1} Fe DM) or moderate (500-3000 mg kg^{-1} Fe DM) (Judson and McFarlane, 1998). Suttle *et al.* (1982) found that Cu absorption was reduced by 50% in ewes supplemented with either a chalky or clay soil containing 2400 and 1400 mg kg^{-1} Fe DM respectively compared to a sandy soil containing 140 mg kg^{-1} Fe DM.

Dietary Fe intakes may also affect Cu absorption according to the presence of S within the soil or feedstuff. The use of high S-containing fertilisers to improve pasture productivity may therefore exacerbate the antagonistic effects of Fe on Cu metabolism (see section 1.4.3.).

1.3. Copper metabolism

Copper metabolism involves the effective absorption, transport, distribution, storage, utilisation and excretion of the element to maintain homeostasis (Mercer, 1997). Differences in Cu metabolism have been found to exist among ruminants; within the same species (Wiener and Woolliams, 1983; Du et al., 1996), between sheep and cattle (ARC, 1980), between sheep and deer (Freudenberger et al., 1987) and in moose and goats (Frank et al., 2000c).

Studies of Cu metabolism have been achieved using nutritional balances, whole animal compositional analyses and radioisotope tracer studies to quantify the fraction of the element that is consumed, absorbed, retained and excreted (Lee et al., 2002). Numerous authors have attempted to represent kinetic models of Cu metabolism using Cu stable isotopes or radioisotopes to represent quantitative 'routes' of Cu flow through the body (Buckley, 1991). Stable isotopes have the advantage over Cu radioisotopes in that they may be studied for longer periods of time. ^{64}Cu in sheep (Weber et al., 1980; 1983), ^{65}Cu in lactating dairy cows (Buckley, 1991) or ^{67}Cu in sheep (Gooneratne et al., 1989c) have previously been used in studies of Cu metabolism. Weber et al. (1980) proposed in a short-term Cu metabolism model in sheep that there were three 'pools' of Cu present. Pool one represented the circulating Cu in the whole blood and pools two and three represented temporary stored compartments of Cu by the liver (before excretion into the bile, or after incorporation into ceruloplasmin). Buckley (1991) represented the metabolism of Cu through the body in more detail using seven Cu stores including the liver, milk, plasma and faeces, and found similar results to Weber et al. (1980) including the small proportion of Cu (<1%) present in the urine after ^{64}Cu administration.

1.3.1. Copper absorption

In most animal species, Cu is poorly absorbed and the extent of absorption is influenced by a number of factors including the chemical form (Underwood, 1977), age of the animal (Suttle, 1975), species and breed (Wiener *et al.*, 1978) and other dietary factors such as antagonistic mineral interactions (as described in sections 1.4.1 to 1.4.3. respectively). Linder (1991) stated that the absorption of Cu appeared to be enhanced by dietary proteins and amino acids and by other chelating agents, possibly from enhanced water solubility. In general, Cu carbonate ($CuCO_3$) and the water-soluble forms, Cu sulphate ($CuSO_4$), Cu nitrate ($Cu(NO_3)_2$ and chloride ($CuCl_2$), are absorbed to a greater extent than Cu oxide (CuO) (NRC, 1985). Copper sulphate is the most standard source of Cu in animal feedstuffs.

The anatomical site of absorption of Cu in ruminant animals occurs primarily in the duodenum (Cousins, 1985) but may also occur to a lesser extent in the jejunum and ileum (Bremner, 1980). The large intestine has also been found to be a significant site for Cu absorption in ruminant animals (Grace, 1975). Differences in the absorption of Cu along the gastrointestinal tract may be due to variations in pH along the lumen which may affect solubility of the Cu in the digesta (Bremner, 1980; Linder, 1991).

The amount of Cu absorbed is partially regulated by the content of Cu within the diet; the efficiency of absorption decreasing with high dietary Cu intakes and increasing when Cu intake is low to maintain homeostasis (Turnland, 1998). Copper has been shown to be absorbed from the mucosal to the serosal side of the gastrointestinal tract by two mechanisms; active transport (Didonato and Sarkar, 1997) and simple diffusion (Bronner and Yost, 1985). Although a specific protein involved in intestinal Cu absorption has not been identified, the Cu importer protein CTR1 (functional in hepatocytes) may be responsible for delivering Cu into the enterocyte (Møller *et al.*, 2000). Another mechanism

of Cu absorption from the small intestine may be via the P-type ATPase, ATP7A (Llanos and Mercer, 2002). P-type ATPases are transmembrane proteins involved in the transport of cations (Mercer, 1997). Once in mucosal cells, a large proportion of Cu is bound to metallothionein (MT). MT's are low molecular weight proteins containing Cu and zinc (Zn) which function in the homeostasis of Cu and Zn, and the subsequent storage, transport and detoxification of metals including Cu, zinc and cadmium (Evering *et al.*, 1991; Kagi, 1991). If Cu is bound to MT, it will remain within the enterocyte. Copper may then enter the portal circulation from the enterocytes via the action of an ATPase, ATP7A (see section 1.3.2.), although this has not been directly demonstrated (Llanos and Mercer, 2002). Within the portal circulation, Cu is bound to carrier proteins, primarily to albumin, whereby it is then transported to the liver (Harris, 1991). Humans with Menkes Disease (MD), an x-linked neurodegenerative disease, have a low uptake of Cu across the small intestine due to a dysfunction of the ATP7A gene, suggesting that ATP7A is involved in the uptake mechanism of Cu into the portal circulation (Danks, 1995). After passing through the enterocytes, Cu then enters the portal circulation where it enters an exchangeable pool of Cu. Here, Cu is bound to albumin, histidine and other amino acids in the Cu^{2+} form (Sarkar, 1999). Cu is then transported in the portal circulation to the liver (Harris, 1991) (see section 1.3.2.).

Genetic differences in metabolism of Cu have been described within and between breeds of sheep (Wiener and Field, 1971; Woolliams *et al.*, 1982; Wiener and Woolliams, 1983) Breeds such as the North Ronaldsay and Texel accumulate more Cu in their liver than Blackface, cross breeds and Finnish Landrace (Woolliams *et al.*, 1982), although the reason for this remains unclear. Cu absorption has also been found to vary between Holstein and Jersey breeds of cattle (Du *et al.*, 1996) and between Simmental and Angus breeds (Gooneratne *et al.* 1994; Ward *et al.*, 1995; Mullis *et al.*, 2003).

The age of the animal is also known to affect Cu absorption. In general, between 5-10% of the Cu in the diet is absorbed by adult ruminant animals. Young milk-fed lambs have been found to absorb 75% of ingested Cu but the weaned lamb may absorb less than 10% of ingested Cu (Suttle, 1975). Bremner and Dalgarno (1973) found that up to 14 weeks of age, calves retained over 50% of the ingested dietary Cu. Therefore, the pre-ruminant animal absorbs Cu as efficiently as monogastric species and more efficiently than the mature ruminant (McDowell, 1992).

Other minerals including zinc (Bremner *et al.*, 1976), sodium (Wapnir and Stiel, 1987) selenium, calcium carbonate, lead, nickel and cadmium (Smith and White, 1997) are also believed to reduce Cu absorption but these complex interactions are beyond the scope of this review of the literature.

1.3.2 Copper transport and cellular uptake

The liver plays a central role in maintaining Cu homeostasis within the hepatocytes by regulating Cu uptake, supplying Cu to endogenous enzymes, incorporating Cu into the Cu-containing enzyme, ceruloplasmin (CP), secreting Cu into the blood for distribution to the brain, tissues, placenta or mammary glands, or may be subsequently excreted in the bile when in excess (Llanos and Mercer, 2002).

Cu uptake within the hepatocyte of humans is via a passive Cu transporter, hCtr1 (Zhou and Gitschier, 1997). Once in the cytoplasm of the hepatocyte, Cu is distributed complexed to a variety of ligands or Cu chaperones, which shuttle Cu to Cu-dependent proteins (Fatemi and Sarkar, 2002) as illustrated in Fig. 1.1.

These Cu chaperones include:

ATOX1 :   *SAH* – ovine homologue of ATOX1 (Lockhart and Mercer, 2000)

Delivers Cu to ATP7B in the transgolgi network (TGN) for inclusion into Cu proteins including ceruloplasmin but may also deliver Cu to partially active ATP7A on TGN for incorporation into lysyl oxidase.

COX17:   Delivers Cu to the mitochondria for incorporation into cytochrome *c* oxidase

CCS: Delivers Cu to Cu-Zn superoxide dismutase (Cu-Zn SOD or SOD_1)

GSH: (Glutathione) Cu chaperone for inclusion of Cu into metallothioneins for metal detoxification
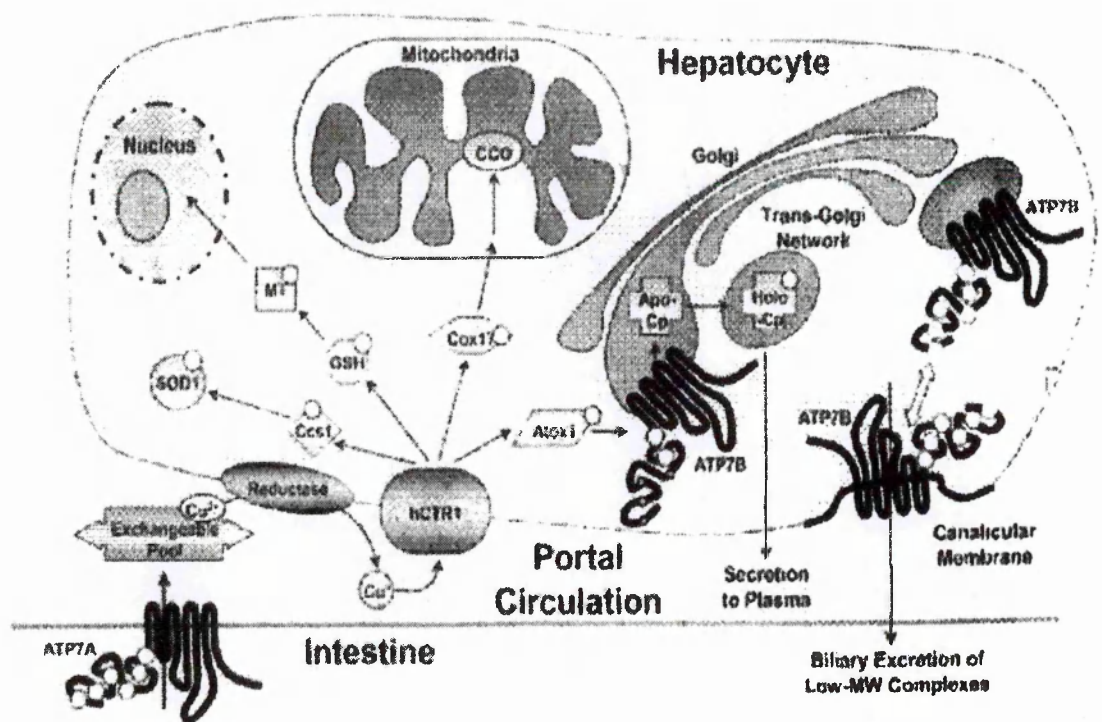
(Fatemi and Sarkar, 2002)

Fig. 1.1.   *Diagrammatic overview of copper transport in the hepatocyte*

10

Human disorders affecting Cu transport were identified by Menkes *et al.*, (1962) (Menkes disease, MD) and Wilson (1912) (Wilson's Disease, WD). These diseases led to the discovery in the 1990's of the Cu 'pumps', the P-type ATPases. The known Cu ATPases, ATP7A (MD) and ATP7B (WD) are key proteins that regulate the flow of Cu in and out of hepatocytes (Llanos and Mercer, 2002). They are highly conserved in diverse organisms ranging from bacteria and yeast to sheep and humans (Llanos and Mercer, 2002).

ATP7A is a Cu ATPase associated with the possible uptake of Cu from the small intestine and also for Cu transport across the blood-brain epithelium (Kodama, 1993). Menkes Disease (MD) is an x-linked neurodegenerative disease due to mutations on the ATP7A protein (Menkes *et al.*, 1962). Human patients are not able to transport Cu from the intestinal cells to the hepatic circulation, resulting in an accumulation of Cu in intestinal cells (Kodama, 1993). In addition, Cu cannot be reabsorbed from the epithelial cells of the proximal tubules in the kidney and therefore, high levels of Cu accumulate in the kidneys (Danks *et al.*, 1972). Patients with MD may also have a defective transport of Cu across the blood-brain barrier, resulting in severe brain Cu deficiency and neural degeneration. Classical symptoms of MD include 'steely hair', hypopigmented hair, neurological defects and/or connective tissue abnormalities (Danks, 1995).

ATP7B is also a Cu ATPase and is expressed primarily in the liver (Schaefer *et al.*, 1999). ATP7B traffics Cu from the cytoplasm of the hepatocyte into apo-ceruloplasmin located in the trans golgi network. It is subsequently incorporated into the holo-ceruloplasmin for secretion into the plasma and supplied to the extrahepatic tissues (Linder, 1991). During periods of Cu excess, ATP7B has been observed to redistribute copper from the trans-golgi network (TGN) to a vesicular compartment, rather than the plasma membrane, where it was proposed to function in the direct excretion of Cu as low molecular weight complexes in to bile by exocytosis (Schaefer *et al.*, 1999). Wilson's disease is an autosomal recessive

inherited Cu toxicity disorder of humans affecting the liver and nervous system (Schilsky and Sternlieb, 1993). Due to the defective mutations of the protein ATP7B protein, Cu cannot be effluxed into the bile or incorporated in to ceruloplasmin (CP), resulting in excessive accumulation of Cu in the hepatocytes in the liver (Schaefer and Gitlin, 1999). WD causes necrosis of hepatocytes and low levels of holo-CP, but normal mRNA CP expression. Classical symptoms of WD include accumulation of Cu in extra-hepatic tissues, the central nervous system, Cu deposits in the eyes (Kayser-Fleischer rings) and dementia (Danks, 1995). Copper storage disorders may also occur in Long-Evans Cinnamon (LEC) rats (Li *et al.*, 1991), toxic milk mouse (Rauch, 1984), Bedlington terriers (Haywood *et al.*, 1996) and West Highland White Terrier dogs (Thornburg *et al.*, 1986).

Ceruloplasmin, a Cu-containing enzyme, is secreted from the hepatocyte into the plasma, whereby CP accounts for between 90-95% of circulating plasma Cu in mammals (Terada *et al.*, 1995). The remaining 5-10% of circulating Cu is loosely bound to albumin and other proteins (Laurie and Pratt, 1986). The function of CP and albumin is to deliver Cu ions to specific organs and tissues (Laurie and Pratt, 1986) (see section 1.5.1.).

1.3.3. Copper distribution and storage

Normal liver Cu concentrations in ruminant animals range from 100 to 500 mg kg^{-1} (DM) although during periods of Cu toxicosis, liver Cu concentrations may range from 2,000 to 3,000 mg kg^{-1} (Dick, 1954). Sheep are susceptible to Cu toxicosis (Lockhart and Mercer, 2000) due to a reduced ability to excrete Cu in the bile, and even moderate dietary intakes of Cu may result in high liver Cu concentrations, resulting in liver failure and possible death (Ishmael *et al.*, 1971). Within the liver, the highest proportion of Cu is distributed in the nuclear fraction (Gooneratne *et al.*, 1979) within the mitochondria, microsomes, cytosol and lysosomes (Kelleher and Ivan, 1986). Distribution within the organelles of the

hepatocyte may vary with age (Lal and Sourkes, 1971), species and Cu status of the animal (Gooneratne *et al.*, 1979). After removal of Cu from the hepatocyte by the Cu ATPase ATP7B, Cu circulates within the blood plasma. Within the plasma, 90-95% of Cu is within CP (Terada *et al.*, 1995) which functions to deliver Cu ions to specific organs and tissues where the Cu is incorporated into intracellular enzymes (Laurie and Pratt, 1986). Copper concentrations of other body tissue vary considerably, but in general, the heart, brain, kidney and intestines contain more Cu than the muscles, spleen, bone and pancreas (Underwood, 1977). Organs which contain very low concentrations of Cu include the endocrine organs, thyroid, pituitary and thymus (Underwood, 1977).

Haywood *et al.*, (1998) investigated the systemic distribution and retention of Cu and Mo from subcutaneous TTM given to Cambridge and North Ronaldsay sheep provided with different levels of dietary Cu. This study found that Cu concentrations significantly increased in the cerebellum and medulla oblongata and were maintained after discontinuation of TTM treatment. In addition, Mo accumulated in all organs including the brain and pituitary gland. This study proposed that some displaced liver Cu may be also redistributed to the brain in a complexed form (Cu-TTM), where it is retained.

1.3.4. Copper excretion

The excretion of Cu from cells is important to control homeostasis. In ruminant animals, bile is the major route of Cu excretion (Evans, 1973; Cousins, 1985) although sheep have a reduced ability to excrete Cu in the bile and will accumulate excess Cu in the liver (Weber *et al.*, 1980). Breed-specific differences in sheep have been suggested to reflect differences in the efficiency of Cu absorption in the gastrointestinal tract rather than differences in the efficiency of biliary Cu excretion (Woolliams *et al.*, 1983). Sheep breeds such as the Texel have a high affinity of absorbing and retaining Cu within the liver, often leading to a Cu toxicity (Woolliams *et al.*, 1982) whereas the Scottish Blackface breed are characterised by their poor ability at absorbing and retaining low levels of Cu within the liver and are

therefore susceptible to hypocuprosis (Woolliams *et al.,* 1986b). The cause of the variation in Cu excretion amongst ruminant animals has not been identified. The Cu ATPase ATP7B functions to remove Cu from the hepatocyte (Bull *et al.,* 1993) and therefore, if the Cu content of the liver is increased, under normal circumstances, ATP7B functions to increase the excretion of Cu into bile (Schaefer *et al.,* 1999; Lockhart *et al.,* 2000). Within the pathway of Cu excretion in bile, van der Sluis *et al.* (2002) reported another protein essential for effective Cu excretion. The protein, MURR1, has been shown to be mutated in Bedlington Terriers (van der Sluis *et al.,* 2002) but is not believed to be the cause of Cu toxicity in North Tyrolean Infantile Cirrhosis in humans (Müller *et al.,* 2003) or North Ronaldsay sheep (van der Sluis, unpublished data). Therefore, differences may occur due to the Cu ATPases within the hepatocyte.

Tetrathiomolybdate (TTM) has been used to treat human patients with WD (McQuaid and Mason, 1990; Brewer *et al.,* 1994) and for treatment of chronic Cu poisoning cases in sheep (Humphries *et al.,* 1986) due to its strong affinity to complex Cu and reduce the harmful effects of Cu toxicosis. Using an animal model of WD, the Long-Evans Cinnamon (LEC) rat, Cu was removed using TTM from the liver and was found to be predominantly excreted in bile (Komatsu *et al.,* 2000). Studies with cannulated sheep also found that the intravenous administration of TTM enhanced biliary Cu excretion (Gooneratne and Christensen, 1984; Symonds and Ke, 1989) and subsequent faecal Cu excretion (Mason *et al.,* 1988). Ke and Symonds (1989) found that in anaesthetised sheep, Cu excretion via bile was increased by 40.7-fold to 21.18 μmol l^{-1} compared with non-treated sheep when given a 100 mg TTM intravenous injection. Similar results were obtained in cattle after intravenous TTM dosing (Gooneratne *et al.,* 1988). High dietary intakes of Mo and S and the subsequent formation of TM within the rumen, may act in a similar way to intravenous TTM by effectively complexing Cu and increasing excretion via the bile. Bile excretion in cattle has found to be several-fold higher than in sheep given similar levels of Mo and S,

which as a result, Gooneratne *et al.* (1994) suggested that cattle are more susceptible to Mo and S induced Cu deficiency than sheep.

Urinary Cu excretion is minimal under normal conditions as most of the circulating blood Cu is bound to CP or within erythrocytes and very little passes the glomerular capillaries (Didonato and Sarkar, 1997). During periods of Cu toxicity or when the biliary route is blocked, Cu excretion may increase via the urine (Symonds and Forbes, 1993).

1.4. Metabolic interactions with other trace elements

1.4.1. Metabolic interactions of copper with molybdenum and sulphur

The biological interactions of Cu, Mo and S within the rumen have been studied extensively and reviewed by several authors (Dick et al., 1975; Clarke and Laurie, 1980; Mason, 1982; Clarke et al., 1987). Molybdenum is readily and rapidly absorbed from most diets (Mills and Davis, 1987) but may strongly inhibit the absorption of Cu in the presence of elevated concentrations of S compounds produced from microbial breakdown, especially from sulphur-containing amino acids or inorganic sources of sulphur (Chidambaram et al., 1984). Sulphur containing compounds are reduced to sulphide and readily combine with Mo in the feed to form a thiomolybdate (TM) complex within the solid phase of the ruminant digesta (Dick et al., 1975; Suttle, 1974a).

TM are produced from a series of compounds by the progressive substitution for sulphate and oxygen in the molybdate anion (MoO_4^{2-}), when hydrogen sulphide and MoO_4^{2-} interact at neutral pH and form a series of mono, di, tri and tetrathiomolybdates (Mills et al., 1978). Tetrathiomolybdate (TTM) is more significant in reducing Cu absorption in ruminant animals than mono, di or tri-thiomolybdates (Suttle, 1974b; Mills et al., 1978). The formation of thiomolybdates, $MoO_nS_{4-n}^{2-}$ (n = 0,1,2,3 or 4) from molybdate and sulphide salts in aqueous media have been studied under conditions which simulate the anaerobic fluid phase in the rumen (Clarke and Laurie, 1980) with the sequential replacement of O^{2-} by S^{2-} (as shown below):

$MoO_4^{2-} + H^+ + HS^- \rightarrow H_2O + MoO_3S^{2-}$ Monothiomolybdate

$MoO_3S^{2-} + H^+ + HS^- \rightarrow H_2O + MoO_2S_2^{2-}$ Dithiomolybdate

$MoO_2S_2^{2-} + H^+ + HS^- \rightarrow H_2O + MoOS_3^{2-}$ Trithiomolybdate

$MoOS_3^{2-} + H^+ + HS^- \rightarrow H_2O + MoS_4^{2-}$ Tetrathiomolybdate (TTM)
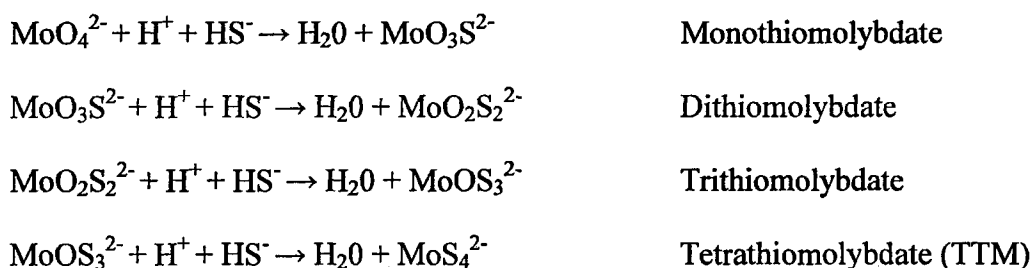
The thiomolybdate complexes bind Cu to form Cu-TM complexes that are not readily absorbed by the animal but are excreted in the faeces. At high Mo intakes (>8 mg kg^{-1} Mo DM) or low Cu:Mo ratios (<1) in sheep, TM leave the rumen in absorbable forms (Price *et al.*, 1987). If Cu availability within the rumen is low, TM complexes may be absorbed into the blood and tissues, where they sequester the Cu from Cu containing enzymes and lower tissue Cu concentrations. This formation and absorption of TM complexes may therefore act systemically and induce clinical symptoms in the ruminant animal (see section 1.6.).

In vitro studies have shown that TTM inhibit Cu-dependent enzyme activities including superoxide dismutase, ceruloplasmin, cytochrome oxidase and tyrosinase (Chidambaram *et al.*, 1984). Mason *et al.* (1982) found that both duodenal and intravenous infusions of tri and TTM produced rapid decreases in plasma ceruloplasmin activity in sheep. Work by Chidambaram *et al.* (1984) suggested that TTM was an irreversible inhibitor of ceruloplasmin, but later work by Kelleher and Mason (1986) and Lannon and Mason (1986) refuted this idea, proving that di, tri and tetrathiomolybdate were reversible inhibitors of ceruloplasmin *in vitro*. The discrepancy between these observations can be attributed to the buffer and pH in which the reactions were carried out, rather than any ambiguity in the inhibition itself (D. Illingworth, personal communication).

Ammonium TTM has been effectively used to treat human patients with Wilson's Disease (WD) (Walshe, 1992) and sheep with chronic Cu toxicity (Gooneratne *et al.*, 1981a). Because of the effective binding capacity of TTM to Cu and the systemic effect of depleting Cu stores, Gooneratne *et al.* (1981a) found that the intravenous administration of TTM was effective in lowering liver Cu levels, and thus, preventing chronic Cu poisoning in sheep. Work by Haywood *et al.* (1993) found that Cu-TTM given to relieve Cu poisoning in a flock of Bleu de Maine sheep was redistributed to the brain and pituitary gland. Additional evidence supporting the claim that TM is absorbed was proved after infusion into the rumen of sheep with [99]Mo-labelled dithiomolybdate and trithiomolybdate.

An insoluble fraction was subsequently found in the plasma of sheep (Mason *et al.*, 1982) and cattle (Hynes *et al.*, 1984). The administration of [99]Mo-TTM either into the rumen or intravenously produced insoluble fractions in the plasma, giving the first conclusive evidence of TM formation and absorption *in vivo* in sheep (Mason *et al.*, 1982).

1.4.2. Metabolic interactions of copper with sulphur

Sulphur is contained in feedstuffs in various inorganic forms (mainly inorganic sulphate) as well as in organic compounds (mainly S-containing amino acids and proteins) (Spais *et al.*, 1968). During digestion, S compounds including organic and inorganic sulphate, are reduced within the reticulo-rumen by micro-organisms to produce sulphides (Lewis, 1954). Increasing dietary S intakes as sulphate or S-amino acids (methionine and cysteine) have been found to reduce Cu absorption due to the formation of insoluble Cu-sulphide complexes in the gastrointestinal tract (Suttle, 1974a), although it is unlikely that S will exhibit significant effects on Cu metabolism independently, compared with the Mo x S interaction. Increased dietary S may increase concentrations of free sulphides within the digestive tract, forming insoluble Cu-sulphide compounds, which are not absorbed, but excreted in the faeces (Suttle, 1974a). Suttle (1986) found that only small increases in herbage molybdenum and sulphur concentrations caused major reductions in Cu availability (Fig. 1.2). Dick (1954) found that by increasing S intakes of sheep from 0.04 to 2.4. g kg^{-1} S, Cu concentration within the liver was reduced by up to 30%. Studies by Grace *et al.* (1997) found contradictory findings to Suttle (1974a) and Dick (1954). When S intakes for grazing lambs were increased from 3.9-7.9 g S day^{-1}, there was no significant effect on Cu status of the lambs when grazing pastures containing similarly low levels of Mo (<0.5 mg kg^{-1} Mo DM) (Suttle, 1974a). Although increasing S intakes reduced Cu entering the duodenum, adequate amounts of Cu were still being absorbed as assessed by liver Cu concentration.
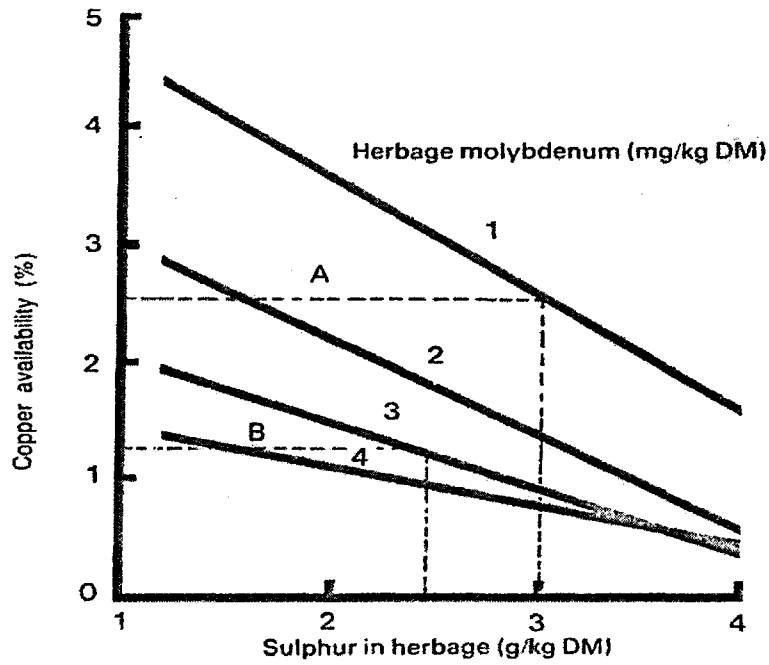
Fig. 1.2   *Estimating the availability of copper in herbage from its molybdenum and sulphur concentrations: the difference of 3 mg molybdenum and 0.5 g sulphur/kg DM between pastures A and B is sufficient to reduce availability from 2.6 to 1.3 per cent, doubling the grazing animals requirement of copper from the pasture.*

(Suttle, 1986)

19

1.4.3. Metabolic interactions of copper with iron

High levels of dietary Fe are known to cause antagonistic interactions with Cu, but less attention has been paid to the precise mechanism as to how dietary Fe may act as a Cu antagonist in comparison to Mo. It has been suggested that high dietary intakes of Fe may complex with S within the rumen and produce an insoluble FeS complex, which binds with Cu but is not absorbed (Suttle et al., 1984). In contrast to TTM, the FeS complex has a high affinity for complexing Cu in the rumen but is not absorbed. Work by Humphries et al. (1983), Bremner et al. (1987) and Phillippo et al. (1987a) found that dietary Fe inclusion to cattle produced no clinical symptoms of a secondary Cu deficiency when compared to Mo supplemented animals. In addition, Phillippo et al. (1987b) also found that Fe supplemented heifers exhibited no alterations in fertility compared to Mo supplemented cattle (See section 1.6.6.). These findings suggest that a FeS complex reduces Cu absorption only within the rumen environment. Combined Fe and Mo supplements to calves were not found to have any more of an additive effect than the antagonists seperately (Humphries et al., 1983).

Copper status, as measured by plasma and liver Cu concentrations in cattle have been found to be reduced by varying dietary intakes of Fe (Standish et al., 1969; Humphries et al., 1983; Bremner et al. 1987; Phillippo et al. 1987a; Chase et al., 2000). Humphries et al. (1983) found that 890 mg kg^{-1} Fe DM was sufficient to lower Cu status in calves, but increasing supplements of Fe had no additional effect on reducing Cu status. The effect of Fe supplementation has been found to greatly reduce liver and plasma Cu levels in weaned calves but not in the pre-ruminant calf (Bremner et al., 1987). This may suggest that differences occur due to the efficiency of absorption depending upon the development of the digestive tract or may be due to the additive effect of S in the developed rumen (Suttle et al., 1984).

1.5. Copper enzymes

Copper is an essential component of a number of proteins and enzymes involved in metabolism (Linder, 1991) and therefore, research has been directed at measuring Cu containing "cupro-enzymes" following Cu deficiency (Prohaska, 1990). These include ceruloplasmin, superoxide dismutase, lysyl oxidase, amine oxidase, cytochrome oxidase and tyrosinase. Most examples of altered enzyme activity following either a primary or secondary Cu deficiency involve lowered enzyme activities. Some of these enzymes are ubiquitous enzymes for Cu regulation in all animal cells, whilst other cupro-enzymes have more specialised functions and are unique to certain cells (Prohaska, 1990).

1.5.1. Ceruloplasmin (CP)

Ceruloplasmin (CP), also known as ferroxidase (EC1.16.3.1), is a blue Cu metalloenzyme with oxidase activity, first described by Holmberg and Laurell (1948). It is a single-chain polypeptide of 1046 amino acids to which several carbohydrate chains are attached (Linder *et al.*, 1998). CP is found in the plasma of most vertebrate species and is synthesised in the liver at a constitutive rate (Gitlin *et al.*, 1992). Under normal conditions, it is secreted in the plasma as holo-ceruloplasmin, where CP accounts for between 90-95% of circulating plasma Cu in mammals (Terada *et al.*, 1995). The amount of Cu per protein molecule has been reported to vary between 5.5 and 7 Cu atoms per molecule for the human protein (Fee, 1975), whilst bovine and ovine CP contain 6-8 Cu atoms per molecule (Calabrese *et al.* 1983). Bovine and ovine CP has a molecular weight of approximately 132 000 daltons and a turnover of two to three days (Linder, 1991).

Ceruloplasmin is necessary for the oxidation of Fe, allowing it to bind with the Fe transport protein, transferrin (McDowell, 1985). The ferroxidase activity of CP enables iron to be mobilised from its stored ferrous state in the liver and intestinal mucosa (as Fe^{2+}) to a ferric state (Fe^{3+}) whereby it is then transported in the bloodstream via transferrin (Frieden,

1970). During periods of Cu deficiency, when CP concentrations fall very low, there is a gradual accumulation of Fe in liver and other organs (Tran *et al.,* 1999). Ceruloplasmin is a polyfunctional protein which is also involved in free radical scavenging and anti-oxidant activity (Goldstein and Charo, 1982), Cu and Fe transport (Linder and Hazegh-Azam, 1996), incorporation of Fe into the storage protein, ferritin (Saenko *et al.,* 1994) and as an important acute-phase reactant involved with inflammation (Linder and Hazegh-Azam, 1996). Ceruloplasmin also acts as a transport protein for Cu, the function being to deliver Cu to the cell membrane receptor (Saenko *et al.,* 1994).

Many other tissues other than the liver have shown CP expression and possibly secrete it, particularly those involved in producing proteins for other body fluids, including sertoli cells, mammary gland, and the choroid plexus of the brain (Linder and Hazegh-Azam, 1996). Ceruloplasmin concentrations are known to fluctuate extensively in numerous diseases and hormonal states, and in man, CP has been utilised diagnostically for a variety of clinical and pathological conditions including rheumatoid arthritis, liver disease, chronic infections and genetic diseases including WD (Scheinberg and Gitlin, 1952) or Menkes Disease (Danks *et al.,* 1972).

In ruminant animals, CP has been used diagnostically as an indicator of Cu status (Mackenzie *et al.,* 1997) (see section 1.7). Ceruloplasmin activity has been found to decrease with nutritional Cu depletion of ruminant animals during a primary Cu deficiency (Blakey and Hamilton, 1985) and during exposure to high dietary intakes of Mo (Humphries *et al.,* 1983). Although the precise mechanism by which CP activity is reduced due to dietary Mo is unknown, the formation of TM complexes within the rumen may act systemically due to the absorption of TM (as their ammonium salts), and subsequently reduce CP activity. Mason *et al.* (1982) found that both duodenal and intravenous infusions of tri and tetra TM produced rapid decreases in CP activity *in vivo* in sheep but TM were

reversible inhibitors of CP activity *in vitro*. An alternative theory may be that TM may directly alter the effectiveness of the Cu chaperone, ATOX1 (see section 1.3.2.) within the hepatocyte, causing a reduction in Cu incorporation into the apo-CP and subsequent decrease in Cu incorporation into the holo-enzyme. The reduction in CP activity during a secondary Cu deficiency requires further investigation.

1.5.2. *Superoxide Dismutase (SOD)*

Superoxide dismutase (SOD) (EC 1.15.1.1.) was first isolated from bovine red blood cells and liver by Mann and Keilin (1938). SOD is a Cu dependent antioxidant enzyme (McCord and Fridovich, 1969) which is ubiquitous amongst aerobic organisms and is essential in catalyzing the convertion of the two superoxide radicles (O_2^-) normally produced during aerobic metabolism to the lesser reactive hydrogen peroxide and oxygen (Fridovich, 1975) and to prevent cellular membrane damage:

$$2\,O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

(Fridovich, 1975)

Two types of SOD are known to exist in eukaryotes. These contain either two atoms of Cu and two atoms of Zn (CuZnSOD) (Bossa *et al.*, 1980) referred to as SOD_1, or one manganese atom per subunit (MnSOD) (SOD_2). SOD_1 is found primarily in the cytosolic fraction of the cell (Chang *et al.*, 1988), whilst SOD_2 is within the mitochondria (Slot *et al.*, 1986). Commercial kit assays for SOD activity do not distinguish between these two isoforms of the enzyme and therefore, the generic term SOD has been used. However, SOD_1 is the predominant isoform and therefore, generic SOD activity may still reflect Cu status.

SOD is enhanced in many intercellular reactions including oxidative stress, during phagocytosis and from the membranes of leucocytes and therefore the role of SOD is to

23

prevent the destruction of membrane lipids and intercellar structures (Sies, 1993). SOD's also prevent the conversion of oxyhaemoglobin to methemoglobin preventing erythrocyte fragility (Lynch *et al.*, 1976; Goldberg and Stern, 1977).

Studies in sheep have indicated that oxidative stress induces or enhances the activity of SOD (Sansinanea *et al.*, 1997). The only factor known to reduce SOD_1 activity in sheep is a primary Cu deficiency (Suttle and McMurray, 1983).

SOD activity has previously been used as an indicator of Cu status in ruminant animals as it has been suggested to be a more useful indicator of Cu status than other current methods as it less sensitive to short-term changes in dietary Cu (Suttle and McMurray, 1983) (see section 1.7).

1.5.3. Amine Oxidase

Amine Oxidase (AO) (EC 1.4.3.6.) is a Cu-dependent enzyme involved with catalysing the oxidative deamination of biogenic amines and the cross-linking of collagen and elastin (Bachrach, 1985). Work by Mulryan and Mason (1987; 1993) and Kelleher and Mason (1979) found that AO activity was strongly inhibited by molybdate and TM *in vitro*. Further work by Mason (1986; 1990) supported the hypothesis that a secondary Cu deficiency may enhance TM production *in vivo* and could directly affect Cu metalloenzymes including AO. Amine oxidase activity in plasma has also been found to decline during a primary Cu deficiency in sheep, cattle and pigs (Paynter, 1987). Plasma AO levels have been found to be extremely variable due to their apparent resistance to reductions in Cu intake and as such, activity declines at a relatively late phase (Claypool *et al.*, 1975).

24

1.5.4. Lysyl oxidase

Lysyl oxidase (EC 1.4.3.13) is a Cu-dependent enzyme present in high concentrations in dense connective tissue including the skin, teeth, heart, bones and cartilage cells and is responsible for the maturation and cross-linking of collagen and elastin. (Rucker *et al.*, 1996; 1998). Lysyl oxidase cross-links polypeptide chains of elastin and also collagen fibres to produce a three dimensional matrix which gives the body elasticity and mechanical strength (Linder, 1991; Rucker *et al.*, 1996). Reduction in lysyl oxidase activity due to a primary Cu deficiency may incur defects including defects in elastic tissue formation, leading to aneurysms and spinal curvature, pulmonary disease, increased proportions of soluble collagen and other defects of connective tissue (collagen and elastin) and bone formation (O'Dell, 1976).

1.5.5. Cytochrome Oxidase

Cytochrome oxidase (COX) or cytochrome *c* oxidase (EC 1.9.3.1) is found primarily in the membranes of the mitochondria (Chance and Leigh, 1977). The enzyme contains two Cu atoms and is the terminal enzyme in the electron transport chain (Green *et al.*, 1956). The enzyme is important for energy generation through oxidative phosphorylation and cell respiration (Linder, 1991). It reduces O_2 with four electrons (from cytochrome *c*) to form two molecules of water. It was confirmed by Okunuki *et al.* (1958) that the enzyme contained Cu, Fe and heme. During periods of primary Cu deficiency, tissue COX activity has been found to be reduced in the sheep (Mills and Williams, 1962), pigs (Gubler *et al.*, 1957) and calf (Mills and Dalgarno, 1970).

Swayback disease, also known as enzoonotic ataxia, is a neuro-degenerative disorder that involves motor in-coordination of the hind limbs in lambs and kids (Howell *et al.*, 1964). In the foetal lamb, Cu levels have been found to be depleted in the central nervous system and the neurons (Mills and Williams, 1962; Fell *et al.*, 1965). A primary Cu deficiency was found to reduce the COX activity in the mitochondria in the brain of diseased sheep (Mills

and Williams, 1962; Smith *et al.*, 1976; Alleyne *et al.*, 1996) and hence, a decreased ATP activity which enhanced abnormalities in the central nervous system (Alleyne *et al.*, 1998). Depletion of COX has been shown to occur in cerebral cortex, brain stem and spinal cord of ataxic lambs (Howell and Davison, 1959; Mills and Williams, 1962). In addition, mitochondria isolated from livers of swayback-diseased animals have revealed an unusual structure and uncharacteristic kinetics in COX (Gallagher *et al.*, 1973; Alleyne *et al.*, 1998). Alleyne *et al.* (1996) found that lambs with swayback had lower than normal COX content of mitochondria, lipid depleted mitochondria and different protein composition of the COX enzyme.

1.5.6. Tyrosinase

Tyrosinase (EC 1.14.18.1) is a Cu-dependent enzyme involved with catalysing the oxidation of tyrosine to the black pigment melanin (Davis and Mertz, 1987). During a secondary Cu deficiency, ruminant animals with dark pigmented coats incur a change in coat or fleece colour (achromotrichia). Lack of pigment production in black fleeced sheep and greying of black or bleaching of brown hair in cattle, especially around the eyes ("spectacles"), are common clinical symptoms observed in ruminant animals (Underwood, 1977). The pigmentation process is so susceptible to changes in Cu concentration, that alternating bands of pigmented and unpigmented wool fibres can be produced accordingly as Cu is added or withheld from the diet (Underwood, 1977). Loss of hair colour in cattle was observed in cattle that received dietary Mo but not in cattle that received dietary Fe even though both types of deficiency were equally as severe as determined by the Cu status of the animal (Phillippo *et al.*, 1987a, b). These findings may have been due to the systemic effect of absorbed TM on the tyrosinase enzyme in the Mo-supplemented cattle.

1.6. Clinical symptoms of a secondary copper deficiency

The systemic effect of absorbed TM may induce clinical symptoms of a secondary Cu deficiency. In animals that do exhibit clinical symptoms, the degree of severity of these clinical symptoms may vary within a species or between species and may be dependent on the Mo content of the diet (Humphries *et al.*, 1983; Phillippo *et al.*, 1987a). The onset of clinical symptoms may be ascribed to the inactivation of Cu-dependent enzymes within the blood due to the antagonistic effect of absorbed TM within the blood and tissues.

Significant findings by Humphries *et al.* (1983) and Phillippo *et al.* (1987a) found that clinical symptoms (including changes in hair texture and colour) and a decrease in production in cattle was more prevalent when Mo was present in the diet compared to dietary Fe. Dietary Fe greatly altered Cu metabolism (as measured by Cu status) but had no effect on clinical appearance or growth. Therefore, alterations in clinical symptoms and performance do not occur when Cu status is low (Phillippo *et al.*, 1987a) but when increased intakes of dietary Mo enhance the formation and absorption of TM complexes resulting in a systemic decrease in Cu availability.

1.6.1. Scouring or diarrhoea

The role of Mo in the development of diarrhoea in ruminants is unknown. Severe diarrhoea or 'scouring' was described by Ferguson *et al.* (1943) in cattle grazing the Mo rich 'teart' pastures in Somerset, UK. Severe diarrhoea in cattle often occurs with a rapid onset in animals grazing pastures containing high Mo concentrations, often leading to lethargy and dehydration and a reduction in performance. Low levels of Cu do not cause diarrhoea to such an extent as when high levels of Mo are present in the diet (Ward, 1978). The differences in susceptibility to diarrhoea in grazing cattle and sheep are unknown. Histochemical and ultrastructural changes have been observed in the small intestinal epithelium of animals with severe diarrhoea (McDowell, 1992).

1.6.2. Depigmentation of hair or wool

The loss of pigment in hair or wool (achromotrichia) is a common characteristic of a secondary Cu deficiency in ruminant animals. Cattle with dark/black coats may exhibit a browning or gingering effect to the coat. Alterations in pigmentation of the hair or wool may be due to the inactivation of the enzyme tyrosinase as described in section 1.5.6. Studies by Moeini (1997) showed that in a series of experiments involving repeated depletion and repletion of dietary Cu to Hebridean sheep presented with diets containing dietary Mo, altering pigmented and unpigmented bands could be identified in the wool fibres due to the inactivation / activation of Cu-containing enzymes. Formation of black hair or wool can be prevented within two days if the level of dietary Cu is low and if dietary Mo and S intakes have been increased (Underwood, 1977). Changes in hair colour were also observed in cattle fed diets containing dietary Mo but not in Fe treated cattle (Humphries et al., 1983; Phillippo et al., 1987a,b).

1.6.3. Altered keratinisation of hair or wool

Altered keratinistaion of wool and hair have been observed in ruminants with a low Cu intake (Suttle and Angus, 1976) but not directly as a result of high intakes of dietary Mo. The polypeptide chains of keratin fibres are cross-linked by disulphide bonds which are formed by the oxidation of –SH groups of the cysteine residues present in the polypeptide chain (Linder, 1991). A lack of 'crimp' in fleece or hair results in a reduction in tensile strength producing straight "steely" wool. Alopecia may occur, elastic properties are abnormal and wool will lie permanently if stretched (Marston, 1946).

1.6.4. Skeletal abnormalities

Copper is essential for maturational cross-linking of elastin and collagen as it is the cofactor for lysyl oxidase (Harris et al., 1980). In the absence of Cu, there is impaired formation of cross-linkages and a reduction in the strength of collagen fibres (Harris et al.,

1980). Skeletal abnormalities due to Cu deficiency have been reported in a variety of species including lambs (Suttle *et al.*, 1972), calves (Suttle and Angus, 1978), farmed red deer (Thompson *et al.*, 1994), moose (Rehbinder and Petersson, 1994) and bison calves (Woodbury *et al.*, 1999). Abnormalities in the skeleton have included osteoporosis (Suttle *et al.*, 1972), reduced osteoblastic activity (Parry *et al.*, 1993), enlargement of the epiphyseal cartilage (Suttle and Angus, 1978), lameness (Irwin *et al.*, 1974) and spontaneous fractures and brittle bones (Cunningham, 1950; Suttle *et al.* 1972). Bone lesions due to a secondary Cu deficiency have been described in cattle (Irwin *et al.*, 1974), sheep (Hogan *et al.*, 1971) and moose (Frank *et al.*, 2000a, d). Parry *et al.* (1993) found that when TTM was provided in diets to Hooded Lister rats, femur length and cell proliferation in the growth plate was markedly reduced compared with Mo-only supplemented rats. The same antagonistic effect of absorbed TM may account for skeletal abnormalities observed in ruminant animals receiving diets containing Mo.

1.6.5. Neonatal Ataxia

Neonatal ataxia, also known as 'swayback' is a nervous disorder observed in lambs, goats and deer (McDowell, 1992). Swayback is a clinical condition resulting from a lack of or degeneration of the myelin sheath in the spinal cord and nerve fibres and is associated with low levels of Cu in the foetal brain and liver (Underwood, 1977). The condition may occur as one of two forms; as an acute form in newborn animals or as a delayed condition, manifesting itself weeks or months after birth. All affected animals show in-coordination of movement, staggering or an inability to stand or walk. The delayed condition has been reproduced experimentally in primary (Suttle *et al.*, 1970) and in secondary Cu deficiency induced by Mo and S in lambs (Suttle and Field, 1969). The role of Cu in neonatal ataxia and the relationship with cytochrome oxidase is described in section 1.5.5.

1.6.6. Fertility

Impaired reproductive performance associated with a secondary Cu deficiency in sheep and cattle have been confirmed by several authors (du Plessis *et al.*, 1999a,b; Phillippo *et al.*, 1987b; Van Niekerk and Van Niekerk, 1989b). Early suggestions of a possible effect of Mo on fertility were by Munro (1957). Cattle grazing Mo rich peat soils in East Anglia that had low fertility rates were significantly improved by appropriate Cu supplementation. Similarly, a Mo-induced subfertility in cattle has been found to be improved with Cu supplementation (Black and French, 2000). Mackenzie *et al.* (2001) showed that treatment with a Cu bolus reduced the insemination to conception rate of dairy cattle from 2.5 to 1.7 for control and Cu-treated animals respectively. Other reports of alterations in reproductive performance due to a secondary Cu deficiency have included embryonic loss (O'Gorman *et al.*, 1987), reduced or absent oestrus (Phillippo *et al.*, 1987b), delayed onset of puberty (Phillippo, 1987b), reduced luteinising hormone release (du Plessis *et al.*, 1999a), reduction in size and function of ovaries (du Plessis *et al.*, 1999a) and lack of signs of behavioural oestrous in sheep (du Plessis *et al.* 1999b). Phillippo *et al.* (1987b) found that heifers supplemented with Mo showed delayed puberty, a reduction in percentage conception rate and a reduction in pulsatile LH release compared to Fe supplemented heifers.

At a herd level, fertility in the UK dairy herd is declining (Royal *et al.*, 2000) although cattle with infertility problems often have normal plasma Cu concentrations (above 12 μmol l^{-1}). Therefore, the systemic effect of absorbed TM may have direct effects on altering the reproductive performance in ruminant animals, although the precise mechanism by which this may occur has not been established. Dietary Mo in the form of TM may exert direct effects on ovarian steroid secretion (Kendall *et al.*, 2003) or impair reproductive hormone release (FSH or LH) (Moffor and Rodway, 1991).

1.6.7. Growth retardation

Reports of Mo or Fe affecting growth rate have produced inconsistent findings within the literature. Most commonly, growth retardation has been associated with low Cu:Mo ratios (<3.0) (Underwood and Suttle, 1999). Moffor and Rodway (1991) found that intravenous TTM reduced live weight gains and delayed the onset of puberty in lambs compared to the respective control groups (268 days versus 245 days respectively). Phillippo *et al.* (1987a, b) found that heifers supplemented with Mo grew significantly slower after 16 weeks on trial compared to control or Fe supplemented heifers. These results are also in accordance with work by Humphries *et al.* (1983) and Smith *et al.* (1975) in calves. Although Fe is known to affect Cu metabolism it has not been shown to have a such a dramatic effect on growth rate as Mo (Humphries *et al.*, 1983; Phillippo *et al.*, 1987a).

1.6.8. Immune function

Cu deficiency has been associated with reduced immune function and increased susceptibility to disease and infection (Stabel and Spears, 1989) although the effects of dietary Mo on different aspects of the immune system have produced inconsistent findings. Woolliams *et al.* (1986a) found that microbial infections produced greater mortality amongst lambs grazing improved pastures compared to Cu supplemented lambs. Calves born to dams fed diets containing Mo were also more susceptible to diseases whilst nursing their dams than calves from Cu adequate cows or cows fed a high Fe diet (Gengelbach *et al.*, 1997).

Numerous techniques have been applied to assess effects on immune responses by dietary antagonists, including lymphocyte proliferation tests (Ward *et al.*, 1993; Arthington *et al.*, 1996), humoral immune responses (Gengelbach and Spears, 1998; Stabel *et al.*, 1993), superoxide dismutase activity (Boyne and Arthur, 1986) and neutrophil phagocytic function (Xin *et al.*, 1991).

Gengelbach and Spears (1998) found that calves receiving dietary Mo had a lowered serum antibody responses to porcine erythrocytes than calves fed a control diet (containing no Cu or Mo). Boyne and Arthur (1986) reported that neutrophils from cattle supplemented with Mo had a reduced ability to kill ingested *Candida albicans* compared to cattle supplemented with dietary Fe or Cu supplemented cattle. Also, neutrophils from steers supplemented with Mo had a reduced ability to kill *Staphlococcus aureus* compared to unsupplemented control animals (Xin *et al.*, 1991).

It is clear from these reports that dietary antagonists, Mo and S, can alter the immune function in ruminants via a Cu dependent mechanism. However, this mechanism has not been fully established although Boyne and Arthur (1986) proposed that this may be attributed to alteration in SOD activity and antioxidant capacity.

1.7. Determination of copper status

Although significant progress has been made over the past fifty years to determine the mechanisms by which dietary Mo or Fe alter Cu metabolism in ruminant animals, accurately determining Cu status still remains problematic.

The most common diagnostic aids currently used to assess Cu status are blood Cu concentrations, including plasma Cu (Pl-Cu) concentration and ceruloplasmin (CP) activity. Normal Pl-Cu reference ranges for ruminant animals are 12-24 μmol l^{-1}, with marginal levels between 8-12 μmol l^{-1} and deficiency levels below 8 μmol l^{-1} (Illingworth, personal communication). However, normal or high Pl-Cu concentrations do not always indicate that the animal has biologically 'available' circulating Pl-Cu as the Cu may be complexed to circulating TM (Suttle, 1980) producing a misleading Cu status of the individual. Pl-Cu concentrations have been found to increase due to high dietary Mo and/or S intakes (Dick, 1953; Bremner and Young, 1978; Van Niekerk and Van Niekerk, 1989a,b) reinforcing the underlying inadequacy of using Pl-Cu concentration independently as a diagnostic test of Cu status.

CP has previously been used as a diagnostic aid as CP accounts for 90-95% of circulating Pl-Cu (Terada *et al.*, 1995) and has been found to be very sensitive to dietary Mo intakes (Humphries *et al.*, 1983). However, CP activity may increase due to infections, stress or pregnancy (Linder, 1991). Therefore, the use of a CP:Pl-Cu ratio was advocated by Mackenzie *et al.* (1997) to be a more useful indicator than Pl-Cu or CP alone to assess the functional Cu status of ruminant animals. No diagnostic test currently takes into consideration TM that may be absorbed into the blood and reduce Cu availability. A theoretical ratio of CP:Pl-Cu of 2:1 (CP in mg/dL and Pl-Cu in μmol l^{-1}) was proposed by Mackenzie *et al.* (1997) for a normal ruminant animal with a ratio of <2.0 indicating that TM was being absorbed into the blood, thus, reducing the availability of Cu in the blood and tissues and reducing the activities of the Cu enzymes. Ratios of 1.0-1.5 may indicate a definite TM problem and <1.0, a serious TM problem. The validity of this ratio was

confirmed by Moeini (1997) and Mackenzie *et al.* (2001) who found that animals diagnosed with low CP:Pl-Cu ratios benefited from Cu supplementation as measured in their responses to improvements in fertility rates. In addition, Kendall *et al.* (2001) reported an increase in the CP:Pl-Cu ratio in cattle with Cu supplementation.

Liver Cu concentration is also commonly used as a diagnostic aid to determine Cu status of ruminant animals but does not take into consideration the effect of dietary Mo and the systemic effects of TM. Increased Pl-Cu concentrations have been observed in association with decreases in liver Cu concentrations (Van Niekerk and Van Niekerk, 1989a). A decrease in liver Cu concentration suggests a homeostatic mechanism by the liver by regulating Pl-Cu concentrations when a high proportion of Cu is bound to TM. Pl-Cu concentration alone is not closely related to liver Cu stores and has been recommended to not be used as the only diagnostic indicator of Cu status (Vermunt and West, 1994). The use of liver biopsies to determine Cu status are also expensive, invasive and time consuming (Minatel and Carfagnini, 2002).

Superoxide dismutase (SOD) has often been used as an indicator for diagnosing Cu deficiency due to the long lifespan of erythrocytes (92 days) and slow decrease in enzyme activity compared to Pl-Cu during Cu depletion (Suttle and McMurray, 1983). Therefore, SOD may be a more useful diagnostic aid indicative of a severe or prolonged Cu deficiency (Paynter, 1987), although little is known about the systemic effects of TM on SOD activity.

Amine oxidase (AMOX) activity has been shown to be induced by a primary deficiency in cattle (Mills *et al.*, 1976). Work by Kelleher and Mason (1979) showed that AMOX activity of CP was inhibited by sodium TTM *in vitro*, with later studies by Mulryan and Mason (1987) showing that AMOX activity was depressed by dithiomolybdate. No known studies to date have investigated the effect of a secondary Cu deficiency on AMOX activity in ruminant animals.

Hair and fleece Cu have been used as diagnostic indicators of Cu status but may only be useful as a diagnostic tool after prolonged periods of Cu deficiency (Suttle and McMurray, 1983). Hair Cu has been found to decline relatively slowly during a primary Cu depletion in bovine (Suttle and Angus, 1976).

1.8.   Conclusion

After extensive reviews of literature on the effects of Mo and Fe on Cu metabolism, it is apparent that there are two divergent views on the aetiology of clinical Cu deficiency. One view, postulated by Suttle (1991) is that the mechanism by which clinical Cu deficiency occurs is due to a lack of Cu, either as a primary deficiency or by Mo, S and Fe preventing absorption in the small intestines. The second mechanism proposed by Phillippo *et al.* (1987a, b) suggested that clinical Cu deficiency was rarely due to a lack of Cu, either as a primary deficiency or due to the prevention of Cu absorption. From their work, Phillippo *et al.* (1987a, b) showed differential effects on clinical signs attributed to Mo and Fe, suggesting that clinical Cu deficiency may have been attributable to toxic systemic effects of TM produced within the rumen and their subsequent absorption. Although the mechanism by which dietary Mo enhances TM formation within the rumen has been established, the systemic effect of absorbed TM on Cu-containing enzymes remains unknown. In addition, variations in responses of dietary Mo or Fe on Cu metabolism remain confusing. Even after extensive research, a reliable diagnostic test to diagnose a secondary Cu deficiency in ruminant animals which takes into consideration absorbed TM still remains to be found, although the CP:Pl-Cu ratio as proposed by Mackenzie *et al.* (1997) requires further validation. A molecular approach to determine how TM may alter expression of Cu-containing enzymes may provide novel and fundamental answers in the pathogenesis of a secondary Cu deficiency. Therefore, this thesis will attempt to investigate the effects of dietary Mo or Fe on Cu metabolism and physiology of sheep, with the aim of producing novel research to help elucidate the mechanism by which these dietary antagonists affect ruminant animals.

Chapter 2

Materials and Methods

2.1. *Proximate Analysis*

All methods were carried out in accordance with the methods of MAFF (1986). All feed samples were analysed in duplicate for content of dry matter (DM), ash, crude protein (CP), ether extract (EE), neutral detergent fibre (NDF) and neutral detergent cellulase gamanase (NCDG) as described in sections 2.1.1 to 2.1.6 respectively.

2.1.1. *Dry Matter*

Dry matter (DM) content was determined on all concentrate feeds by oven drying 100 g of sample into a previously weighed aluminium tray at 100°C until a constant weight was achieved. The content of dry matter was then calculated from the equation:

$$\left\{ \frac{\text{Weight of dry sample (g)}}{\text{Weight of original sample (g)}} \right\} \times 1000 = \text{DM (g kg}^{-1})$$

2.1.2. *Ash*

Ash content was determined by accurately weighing 1 g of dried concentrate feed into a pre-weighed porcelain crucible and then ashing in a muffle furnace (Gallenkamp Muffle Furnace, size 3, GAFSE 620) to 450°C for 16 hours. The ash remaining in the crucible was then cooled in a dessicator to room temperature and reweighed. The content of ash was then calculated from the equation:

$$\left\{ \frac{\text{Weight of ash (g)}}{\text{Weight of original sample (g)}} \right\} \times 1000 = \text{Ash content (g kg}^{-1}\text{ DM)}$$

2.1.3 *Crude Protein*

Crude protein (CP) was determined using an automated Kjeldahl digestion procedure on a decator 1035 autoanalyser (Foss UK Ltd, Oxon, UK). Samples weighing approximately 1 g were placed in folded nitrogen-free Whatman filter papers (Whatman no 1) and placed in clean Kjeldahl tubes. Two kjeltab catalyst tablets (3.5 g K_2SO_4 and 0.4 g $CuSO_4.5H_2O$; Thompson and Capper Ltd.) and 20 ml of concentrated sulphuric acid (6N) was added to each tube and then all tubes were transferred to a digestion block and boiled at 420°C for 45 minutes in a fume cupboard. Samples were then cooled for 20 minutes and 75 ml of deionised water added to each tube. All tubes were placed individually in the auto analyser and digests were made alkali by the addition of 50-65 ml sodium hydroxide. All samples were distilled in a solution of 4% boric acid before titration with 0.2M hydrochloric acid performed by the Kjeltec 1035 autoanalyser (Foss UK Ltd, Oxon, UK). The nitrogen content and crude protein content of the sample (calculated using the sample weight and the volume of acid required to neutralise the ammonia) was calculated from the equation :

1ml of 0.1M HCl = 0.0014g Nitrogen

$$\text{Nitrogen (g kg}^{-1}) = \left\{ \frac{1.4 \text{ x volume of HCl}}{\text{Sample weight (g)}} \right\}$$

Nitrogen (g kg^{-1}) x 6.25 = Crude protein content (g kg^{-1})

2.1.4. *Ether extract*

Ether extract (EE) was determined by weighing approximately 1 g DM of feed into a pre-weighed cellulose extraction thimble (Whatman International Ltd., Maidstone, UK). The thimble was then plugged with de-fatted cotton wool and placed in the extraction unit (Tecator Soxtec 1043, Foss UK Ltd., Oxon, UK). Extraction cups were weighed and placed into the cup holder. Total fat was extracted by boiling samples in 25 ml of petroleum ether (solvent) for 1 hour. Samples were removed from the solvent and rinsed for a further 15 minutes. Final traces of petroleum ether were evaporated off, the extraction

cups were removed from the apparatus, cooled in a fume cupboard and re-weighed when cool.

Ether extract content was calculated from the equation:

$$EE\ (g\ kg^{-1}\ DM) = \left\{ \frac{(\text{extraction cup} + \text{fat weight}) - (\text{extraction cup weight})}{(\text{thimble} + \text{sample weight}) - (\text{thimble weight})} \right\} \times 1000$$

2.1.5. Neutral Detergent Fibre

Neutral detergent fibre (NDF) content of the feed was determined using the method of Van Soest *et al.* (1991). NDF reagent was previously prepared using 93 g disodium ethylene diamine tetra-acetate dihydrate (EDTA) and 34 g sodium borate dissolved in distilled water using gentle heating. One hundred and fifty g sodium lauryl sulphate and 50 ml 2-ethoxy ethanol were then added. In a separate flask, 22.8 g of anhydrous disodium hydrogen phosphate was dissolved in distilled water. The two solutions were then mixed and diluted to 5 litres with distilled water. The pH was adjusted to pH 6.9-7.1.

Approximately 0.5 g ground sample was weighed into a previously dried and weighed Fibretec crucible (P1). The crucible was then secured in the Fibretec apparatus (Tecator Fibretec 1020, Foss UK Ltd., Oxon, UK) and 25 ml of cold NDF reagent added to each sample as well as 0.5 ml Octanol (anti-foaming agent). All samples were brought to boiling point and then digested for a further 30 minutes at a reduced temperature. The heat was then turned off and a further 25 ml of cold NDF reagent added to each crucible and 2 ml α-amylase solution (BDH Laboratory Supplies, Poole, Dorset, UK) (2 g α-amylase was dissolved into 90 ml of distilled water, filtered and 10 ml 2-ethoxy ethanol added and stored at 4°C). The samples were then brought to boiling point and digested for a further 30 minutes at a reduced heat. The heat was then turned off and the samples washed (vacuum filtered) three times using 20 ml of hot (80°C) distilled water. After filtration, a further 25 ml of distilled water and 2 ml of α-amylase were added to each crucible and allowed to stand for 15 minutes before being washed (vacuum filtered) three times with hot distilled

water (80°C) and finally with 20 ml of acetone. All crucibles were removed from the Fibretc apparatus and oven dried at 100°C overnight, cooled in a dessicator and reweighed. Crucibles were then placed in a muffle furnace (Gallenkamp Muffle Furnace, Size 3, GAFSE 620) and ashed at 550°C for 4 hours, cooled in a dessicator and reweighed. The NDF content of the feed was calculated as:

$$NDF\ (g) = (crucible + dry\ fibre\ weight) - (crucible + ash\ weight)$$

$$NDF\ (g\ kg^{-1}\ DM) = \left\{ \frac{NDF\ weight\ (g)}{Sample\ weight\ (g)\ DM} \right\} \times 1000$$

2.1.6. Neutral cellulase gamanase digestibility

Neutral cellulase gamanase digestibility (NCDG) was determined according to the method of MAFF (1986). The same methodology was performed on the feed samples using the method described in section 2.1.5. After the final washing (vacuum filter) stage with hot (80°C) distilled water and the addition of 20 ml acetone, the crucibles were removed from the Fibertec apparatus. Subseals were moistened in distilled water and carefully placed into the bottom of each crucible. To each crucible, 25 ml of cellulase/gamanase solution was added (buffered cellulase solution prepared by weighing 20 g cellulase, (BDH Laboratory Supplies, Poole, Dorset, UK) and 0.1 g of chloramphenicol (Sigma- Aldrich, New Road, Gillingham, Dorset, UK) into a 2 litre wide necked flask. One litre of buffer solution was added (1.36 g sodium acetate dissolved into 500 ml of distilled water, with 0.6 ml glacial acetic acid and diluted to 1 litre, adjusted to pH 4.8), shaken and incubated for approximately 1 hour until dissolved). Cellulase solution and gamanase (Novo Nordisk, 2880 Baysvaerd, Denmark) were mixed in the proportions 9:1 to give a cellulase solution containing 10% gamanase. Suba-seal caps were placed on top of each crucible and shaken to mix crucible contents. All crucibles were then incubated at 40°C for 24 hours, shaking frequently. The cap and suba-seal were removed and each crucible was placed in the Fibretec apparatus and washed (vacuum filtered) three times with hot (80°C) distilled water and once with 20 ml of acetone. All crucibles were removed from the Fibretc apparatus

40

and oven dried at 100°C overnight, cooled in a dessicator and reweighed. Crucibles were then placed in a muffle furnace (Gallenkamp Muffle Furnace, Size 3, GAFSE 620) and ashed at 550°C for 4 hours, cooled in a dessicator and reweighed. NCGD was determined using the indigestible organic matter (IOM) and the ash in the sample:

IOM (g) = (crucible wt + dry fibre wt) - (crucible wt + ash wt)

$$IOM\ (\%) = \left\{ \frac{IOM\ wt\ (g)}{Sample\ weight\ (g)} \right\} \times 100$$

Ash (g) = (crucible wt (g) + ash wt (g)) – (crucible wt (g))

$$Ash\ (\%) = \left\{ \frac{ash\ wt\ (g)}{Sample\ wt\ (g)} \right\} \times 100$$

NCGD (%) = 100 – (IOM (%) + ash (%))

2.2. *Blood sample collection*

Blood samples were collected from lambs or wethers via jugular venepuncture using a 20 gauge 1.5" needle (Becton Dickinson Vacutainer Systems, Plymouth, UK) into 10 ml vacutainer tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK). Tubes containing the anticoagulant heparin were used for collection of whole blood products haematocrit (Hc), haemoglobin (Hb) or erythrocyte superoxide dismutase (SOD) activities and plasma-copper concentration after centrifugation. Silicone coated tubes were used for collection of serum and subsequent analysis of ceruloplasmin (CP) and amine oxidase (AMOX).

Following haematocrit determination, the remaining blood samples collected in heparin tubes were immediately centrifuged at 1000*g* for 15 minutes (Beckman Avanti 30 centrifuge). The supernatant was pipetted off with disposable pipettes and stored in 7 ml bijou tubes (Sarstedt Ltd., Leicester, UK) for storage at -20°C. Blood samples collected in no additive, silicone coated tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK) were refrigerated at 4°C and left to coagulate overnight. All tubes were subsequently centrifuged at 1000*g* for 15 minutes (Beckman Avanti 30 Centrifuge), and the supernatant pipetted off with plastic disposable pipettes and aliquated into micro-centrifuge tubes and the remainder stored in 7 ml bijou tubes (Sarstedt Ltd., Leicester, UK) for storage at -20°C. Prior to analysis, all serum and plasma containing tubes were left to defrost to room temperature and vortexed using a MT-20 vortex-mixer (Philip Harris Ltd., Shenston, UK) to provide a uniform sample.

2.2.1. *Haematocrit*

Haematocrit (Hc) was determined using fresh whole blood. Heparinised capillary tubes (10 x 100mm) (Hawksley and Sons Ltd., Marlborough Road, Lancing, Sussex, UK) were filled with heparinised blood and sealed at one end with Cristaseal (Hawksley and Sons Ltd., Marlborough Road, Lancing, Sussex). They were then placed in a micro-haematocrit centrifuge (Centurion Scientific Ltd, Weston-Super-Mare, Somerset, UK). All capillary

tubes were centrifuged at 3000g for 15 minutes. The haematocrit (%) was determined on a micro-haematocrit reader (Hawksley and Sons Ltd, Marlborough Road, Lancing, Sussex), with the haematocrit being the proportion of packed cells as a percentage of the total volume.

2.2.2. Haemoglobin (manual method)

Haemoglobin (Hb) concentration was determined for all lambs as described in chapter 3 (Procedure number 525 : Sigma Aldrich Ltd., St. Louis, USA). Working standards were prepared by accurately pipetting and mixing thoroughly into 10 ml round bottomed glass test-tubes Drabkins reagent (product no. 525-2) (100 parts sodium bicarbonate, 20 parts potassium ferricyanide and 5 parts potassium cyanide, plus an additional 0.5 ml 30% Brij-35 solution to make Drabkins solution), cyanmethemoglobin (reconstituting hemoglobin standard no. 525-18 with 50 ml Drabkins solution) and hemoglobin standard (product no. 525-18, Sigma Aldrich Ltd., St. Louis, USA) used as an internal standard for all samples at a known concentration of 18 g/dL. A calibration curve was determined by accurately pipetting haemoglobin standard into plastic micro cuvettes and reading the absorbance using a Unicam 8625 Ultra Violet spectrophotometer (Unicam Ltd., York St., Cambridge, UK) at a wavelength of 530-550 nm using the given concentrations:

Tube	Cyanmethemoglobin standard solution (ml)	Drabkins Solution (ml)	Blood Hb (g/dL)
1	0.0	6.0	0.0
2	2.0	4.0	6.0
3	4.0	2.0	12.0
4	8.0	0.0	18.0

Haemoglobin concentrations of all samples were determined from a calibration curve of absorbance values (nm) against blood haemoglobin levels (g/dL). Intra assay coefficient of variations were calculated and described in chapter 3. Hb concentration was determined as g/dL.

2.2.3. Haemoglobin (Cobas Mira Plus Method)

Haemoglobin (Hb) analysis for all animals as described in chapters 4 to 6 inclusive was determined using a Total Hemoglobin kit (procedure no. 525, Sigma-Aldrich Ltd., St Louis, USA) adapted for the Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). Whole blood samples were defrosted to room temperature and vortexed using a MT-20 vortex-mixer (Philip Harris Ltd., Shenston, UK). Into a 1 ml micro-centrifuge tube, 20 μl of whole blood was accurately pipetted into 180 μl red cell lysing agent (Sigma-Aldrich Ltd., St Louis, USA) and vortexed using a MT-20 vortex-mixer (Philip Harris Ltd., Shenston, UK). Samples were then carefully transferred into Mira cups (ABX Diagnostics, Shefford, Bedfordshire, UK), and placed into reagent racks and analysed by an automated method on the Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). For control of precision and accuracy, pooled whole blood was stored in frozen micro-centrifuge tubes at -20°C and defrosted to room temperature and subsequently used as an internal standard against all samples. Intra assay coefficient of variations were calculated and described in respective chapters where appropriate. Haemoglobin concentrations were determined as g/dl.

2.2.4. Ceruloplasmin activity

Ceruloplasmin (CP) activity was determined using an adapted method of Henry *et al.* (1974) for use on the Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). The ability of CP to act as a general oxidase is utilised in this method, where CP will oxidise p-phenylenediamine (PPD) to produce purple products that have an absorption peak between 530-550 nm. As PPD is also oxidised by any copper or iron present in serum, a blank (CPB) must be run in which sodium azide inhibits the CP activity, and the results subtracted from the test (CPT). Individual serum samples were pipetted into Mira cups (ABX Diagnostics, Shefford, Bedfordshire, UK) and placed in the required reagent rack on the Cobas Mira Plus. A 0.1M solution of PPD (BDH Laboratory Supplies, Poole,

Dorset, UK) was prepared in 100 ml of 0.1M acetate buffer and adjusted to pH 6.0. Sodium azide (BDH Laboratory Supplies, Poole, Dorset, UK) was prepared using 0.1% solution in pH 6.0 acetate buffer. A test (CPT) reagent was prepared by adding 20 ml acetate buffer pH 6.0 and 10 ml PPD solution pH 6.0. A blank (CPB) reagent was prepared by adding 10 ml acetate buffer pH 6.0, 10 ml PPD solution pH 6.0 and 10 ml sodium azide solution. The activity of the ceruloplasmin (mg/dl) was calculated as:

$$CPT - CPB = Ceruloplasmin\ activity\ (mg/dl)$$

For control of precision and accuracy, pooled serum was stored in frozen micro-centrifuge tubes at -20°C and defrosted to room temperature and subsequently used as internal standards against all samples. Intra assay coefficient of variations were calculated and described in respective chapters where appropriate.

2.2.5. Superoxide Dismutase activity

Superoxide dismutase (SOD) activity was determined using an adapted method of Misra and Fridovich (1977) for use on the Cobas Mira Plus (Ransod SD125, Randox Laboratories, County Antrim, UK). This method employed xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction. Frozen heparinised blood samples were defrosted (to enhance lysing of cells) and vortexed using a MT-20 vortex-mixer (Philip Harris Ltd., Shenston, UK). Into a 1 ml micro-centrifuge tube (Sarstedt Ltd., Leicester, UK), 250 µl of whole blood was accurately pipetted and a further 750 µl of purite water was added. The sample was vortexed and 10 µl of this sample was then added to 490 µl of 0.01 mol l^{-1} phosphate buffer, pH 7.0 (Ransod, Randox laboratories, County Antrim, UK) into a micro-centrifuge tube and vortexed thoroughly. Samples were then carefully transferred into Mira cups (ABX diagnostics, Shefford, Bedfordshire, UK), and

placed into reagent racks and analysed by an automated method on the Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK).

SOD activity (U/g Hb) was calculated as:

$$\left\{ \frac{\text{SOD units/ml of whole blood}}{\text{g haemoglobin/ml}} \right\} = \text{SOD units/g haemoglobin}$$

For control of precision and accuracy, pooled whole blood was stored in frozen micro-centrifuge tubes at -20°C and defrosted to room temperature, to be subsequently used as internal standards against all samples. Intra assay coefficient of variations were calculated and described in respective chapters where appropriate.

2.2.6. Plasma Copper concentration

Plasma copper (Pl-Cu) concentrations were determined on a Pye Unicam SP9 Atomic Absorption Spectrophotometer with a 10 cm slot burner head using background correction for copper at a wavelength of 324.8 nm. Plasma samples were defrosted to room temperature and thoroughly mixed using a MT-20 vortex-mixer (Phillip Harris Ltd., Shenston, UK) and 1:10 dilutions were prepared using purite water. Samples were vortexed and read on the atomic absorption spectrophotometer. For control of precision and accuracy, stock plasma samples were stored in frozen bijou tubes at -20°C and defrosted to room temperature and used as an internal standard against all samples. Working standards were prepared by dilution of a copper sulphate solution (1000 ppm Spectrosol, BDH Laboratory Supplies, Poole, Dorset, UK) in purite water using a standard curve. Intra assay coefficient of variations were calculated and described in respective chapters where appropriate. Plasma copper concentration was determined as μmol l^{-1}.

2.2.7. Serum Amine Oxidase activity

Serum amine oxidase (AO) was determined using an adapted method of Mulryan and Mason (1992) for use on the Cobas Mira Plus automated analyser (ABX Diagnostics, Shefford, Bedford, UK). This method employed p-dimethylaminobenzylamine as a substrate and one unit of AO activity was defined as the amount of enzyme required to produce an absorbance change of 1×10^{-3} OD (355nm) per minute.

Individual serum samples were carefully pipetted into Mira cups (ABX Diagnostics, Shefford, Bedfordshire, UK) and placed in the required reagent rack on the Cobas Mira Plus. A 0.1M potassium phosphate buffer was prepared by dissolving 1.36 g of KH_2PO_4 in 80 ml deionised water, adjusted to pH 8.0 with 1.0M NaOH and made up to 100 ml with deionised water. Amine oxidase activity was measured using p-dimethyl-aminobenzylamine solution (3.6mM) mixed in with 0.1M potassium phosphate buffer, pH 8.0 at 25°C. The reaction was started with the addition of 28 µl of serum to 252 µl of reagent (1:10 dilution). The product p-dimethylaminobenzaldehyde was measured at a wavelength of 355 nm for 17 minutes.

One unit of AO activity was defined as the amount of enzyme required to produce an absorbance change of 1×10^{-3} (OD 355nm) per min at 25°C:

$$\text{Units/l serum} = \left\{ \frac{\Delta \text{absorbance/min}}{1 \times 10^{-3}} \right\} \times \left\{ \frac{\text{Total vol. of reaction mixture}}{\text{Vol. of serum used}} \right\}$$

For control of precision and accuracy, stock serum samples were stored in frozen bijou tubes at -20°C and defrosted to room temperature and used as an internal standard against all samples. Intra assay coefficient of variations were calculated and described in respective chapters where appropriate.

2.2.8. Liver copper determination (atomic absorption method)

Livers were collected from all lambs as described in chapter 3 from a commercial slaughterhouse. Fresh liver samples were collected from all lambs and placed into clean plastic bags and stored at -20°C for subsequent analysis. Liver copper concentration was determined using the method of AOAC (1990) using a Smith-Hieftje 1000 atomic absorption spectrophotometer (Thermo Jarrell Ash Corporation, Franklin, MA, USA) with a 10 cm burner head and using background correction at a wavelength of 324.8 nm.

All glassware was washed thoroughly: 0.1M HCl (BDH Laboratory Supplies, Poole, Dorset, UK) for two days, rinsed thoroughly in purite water, a further two days rinsing in 0.1M HNO_3 (BDH Laboratory Supplies, Poole, Dorset, UK) and a final thorough rinse in purite water, drained and oven dried.

Frozen livers were defrosted to room temperature and a 1 g section of liver was accurately weighed out and placed into a 15 ml plastic screw capped digestion tube (Sarstedt Ltd., Leicester, UK). Into each tube, 5 ml of concentrated HNO_3 (69% ANALAR: BDH Laboratory Supplies, Poole, Dorset, UK) was carefully pipetted, the screw cap tightened and all tubes placed in a ventilated oven at 60°C for 12 hours until complete digestion had occurred. After 12 hours, all tubes were removed from the oven and the lids removed in a fume cupboard and allowed to cool to room temperature. Samples were carefully transferred into a 25 ml volumetric flask and made up to volume using purite water. Working standards were prepared by dilution of a copper sulphate solution (1000 ppm Spectrosol, BDH Laboratory Supplies, Poole, Dorset, UK) in purite water. Samples were analysed on the atomic absorption spectrophotometer (AAS) and a stock liver sample used as an internal standard against all samples for precision and accuracy. Intra assay coefficient of variation was calculated as described in chapter 3. Liver copper concentration was determined in mg kg^{-1} DM.

2.2.9. Trace element determination of tissue samples (ICP-MS)

Tissue samples obtained in chapter 5 were collected from major organs including liver, cerebellum, ovary and pituitary gland at the slaughterhouse. Fresh liver samples were collected from lambs and wethers as described in chapters 3 to 6 inclusive, into clean plastic bags and stored at -20°C for subsequent analysis. The brain was removed for necropsy according to the procedure of McCurnin and Bassert (2002) and separated into four parts using sterile scalpel blades for cerebrum, cerebellum, hypothalamus and medulla oblongata. The cerebellum, pituitary gland and ovary were stored in 10% buffered formalin for tissue preservation (HD Supplies, Aylesbury, Bucks, UK) and subsequently analysed for trace element determination.

All 28 ml pyrex glass tubes and glass marbles were washed thoroughly: 0.1M HCl (BDH Laboratory Supplies, Poole, Dorset, UK) for two days, thoroughly rinsed in purite water, a further two days rinsing in 0.1M HNO_3 (BDH Laboratory Supplies, Poole, Dorset, UK) and a final thorough rinse in purite water, drained and oven dried.

Livers were thoroughly defrosted and triplicate samples of approximately 0.5 g liver were accurately weighed and transferred into individual bijou tubes (Sarstedt Ltd., Leicester, UK). These were then transferred into a freeze drier (Edwards Modulyo, Crawley, West Sussex, UK) for 72 hours. Dried samples were then weighed again for dry matter determination and transferred into a clean 28 ml pyrex glass tube. Two ml of concentrated HNO_3 was pipetted into each pyrex tube containing the liver sample, covered with a glass marble and heated at 70°C in a ventilated oven for 12 hours until complete digestion had occurred. All samples were allowed to cool and subsequently made up to 10 ml in volumetric flasks using purite water. For pituitary gland, ovary and cerebellums, approximately 0.4 g, 0.8 g and 0.6 g respectively of tissue sample was used with 2 ml concentrated HNO_3 and made up to 10 ml final volume in a volumetric flask with purite water. Further dilutions were prepared where appropriate. Using an Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS) (Elan 6100, Perkin Elmer Instruments,

49

Norwalk, CT, USA), copper, molybdenum, iron, and zinc concentrations were obtained (parts per million, ppm). Merck standards were prepared and a Bovine Liver Standard Reference Material (1577b) (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for precision and accuracy.

2.3. Trace element determination of feeds

Trace element composition of all basal diets in chapters 3 to 6 respectively were determined by ICP-Emission Spectroscopy (Track-scan, Thermo-Jarrell Corporation, USA) following concentrated nitric acid-hydrogen peroxide digestion of all samples. All samples were analysed by a commercial laboratory (Frank Wright Ltd., Ashbourne, Derbyshire, UK).

2.4. Liveweight determination

Lamb weights were recorded once weekly as specified for each experimental chapter using a standard operating procedure with weigh scales (IAE Scales, Leek, Staffordshire, UK), and calibrated using metric standard weights (F.J. Thornton and Co. Ltd., Wolverhampton, UK) for precision and accuracy.

Chapter 3

The effect of dietary molybdenum or iron on copper status and performance of growing lambs

3.1. Introduction

Secondary copper (Cu) deficiency in ruminant animals is caused by high dietary intakes of molybdenum (Mo) and sulphur (S) (Suttle, 1991). Within the anaerobic conditions of the rumen, Mo and S complex to form a series of thiomolybdates (TM) which were first implicated in the aetiology of a secondary Cu deficiency by Suttle (1974b) and Dick *et al.* (1975). TM complexes are known to have a high affinity for Cu, and as a consequence, may form CuTM within the digestive tract of the animal, preventing absorption from the small intestines (Dick *et al.*, 1975; Mason, 1986; Mason *et al.*, 1982; Mills *et al.* 1978). If insufficient dietary Cu is available in the rumen, TM may be absorbed as their ammonium salts into blood and tissues, leading to a systemic reduction in Cu available for Cu-dependent enzyme activities. It is the systemic effect of absorbed TM that affects Cu metabolism and may not be a reduction in Cu availability for absorption that may ultimately produce clinical symptoms. Clinical symptoms attributable to dietary Mo have included changes in coat texture and colour and reductions in growth rate (Humphries *et al.*, 1983; Phillippo *et al.*, 1987a), skeletal abnormalities (Humphries *et al.*, 1983) and acute diarrhoea (Ferguson *et al.*, 1943). Dietary Mo has also been shown to have significant effects on fertility in cattle (Phillippo *et al.*, 1987b) and sheep (Van Niekerk and Van Niekerk, 1989b; du Plessis *et al.*, 1999a, b).

Early studies by Munro (1957) and Littlejohn and Lewis (1960) suggested that there was no correlation between fertility and Cu status in dairy cattle (as measured by serum Cu status alone), as serum Cu concentration and fertility rate were highly variable. Phillippo *et al.* (1982) confirmed studies of Case *et al.* (1973) by suggesting that dietary Mo, rather

than Cu deficiency *per se*, may be the cause of poor reproductive performance in cattle. Dietary Mo was found to significantly reduce the release of luteinizing hormone, reduce conception rate, delay the onset of puberty and cause an increase in anoestrous and anovulation in cattle compared to control or iron (Fe) supplemented cattle (Phillippo *et al.*, 1987b).

The onset of clinical symptoms have also been found to vary within individual animals and within herds. Wentink *et al.* (1999) found that heifers supplemented with saturated $CuSO_4$ solution in water (1 g $CuSO_4$/kg DM) and respective control heifers had very low blood Cu levels (<3 μmol l^{-1}) and liver Cu concentrations (<6 mg kg^{-1}) although no animal showed any clinical symptoms, normally presumed to be indicative of a Cu deficiency. Humphries *et al.* (1983) also found that both dietary Mo and Fe reduced Cu concentrations in the plasma, liver and Cu-containing enzymes, although only animals that received dietary Mo produced clinical symptoms.

The antagonistic effect of dietary Fe in reducing Cu availability has been well documented in cattle (Humphries *et al.*, 1983; Bremner *et al.*, 1987; Phillippo *et al.*, 1987a), although the mechanism by which Cu absorption is reduced due to high Fe intakes is believed to be different to the Cu x Mo x S interaction. Work by Phillippo *et al.* (1987a) found that four month old calves supplemented with 500 mg kg^{-1} Fe had reduced plasma and liver Cu concentrations after 32 weeks on trial, but there was no reduction in growth rate or clinical symptoms as seen in calves fed the same basal diet containing 5 mg kg^{-1} Mo.

Therefore, accurately diagnosing the true Cu status of a ruminant animal has proven to be problematic as there is often a lack of association between Cu status and clinical symptoms. Current methods of assessing Cu status in ruminant animals include plasma Cu (Pl-Cu) or liver Cu concentrations but these methods do not take into consideration the

effect of dietary Mo and the subsequent reduction in Cu availability due to TM. Rather, they define total Cu within the plasma or liver alone and may not provide a true indication of the Cu status of the individual. In cattle, low blood Cu concentrations may indicate a Cu deficiency, however, normal or high Cu concentrations do not necessarily indicate that the animal has a sufficient amount of Cu (Claypool *et al.*, 1975). Unfortunately, at present there is no blood test that is currently available to determine whether Pl-Cu is biologically available or not (Herdt *et al.*, 2000). The sensitivity of the Cu-dependent enzyme, ceruloplasmin (CP) has frequently been used as an indicator of Cu status (Humphries *et al.*, 1983) as CP accounts for 90-95% of plasma Cu (Terada *et al.*, 1995). Therefore, a CP to Pl-Cu ratio (CP:Pl-Cu) for ruminant animals was proposed by Mackenzie *et al.* (1997) as being a more useful biochemical indicator of Cu status than other current methods when assessing animals suffering from TM problems. This ratio assumed that Cu present in CP accounted for approximately 90% of Pl-Cu. The model predicted an ideal CP:Pl-Cu ratio of 2:1, postulating that a ratio less than 2:1 may indicate free TM being absorbed into the blood, which may reduce Cu availability for subsequent metabolic purposes and Cu-dependent enzyme activity. The ratio could potentially identify herds with TM problems and identify animals that may benefit from Cu supplementation.

Therefore, the aim of this study was to investigate the effect of the dietary antagonists, Mo or Fe on Cu status and performance of growing lambs. Copper status was assessed using current diagnostic techniques, including Pl-Cu, CP, superoxide dismutase, amine oxidase and liver Cu concentration in addition to determining the effects of Mo and Fe on the CP:Pl-Cu ratio.

3.2. Materials and methods

3.2.1. Experimental Design and Animals

Fifty-four Charollais x (Suffolk x Mule) female lambs of approximately 10 weeks of age and with an initial mean live weight of 23.2 kg (s.e.d. 0.89) were used in this study. Six representative lambs were slaughtered at the start of the trial to assess liver Cu concentration. The remaining 48 lambs were allocated by live weight to one of four dietary treatments (twelve lambs per treatment) (see section 3.2.2.) using a completely randomized design for a period of ten weeks. Lambs were housed in individual pens on elevated metal floors in a ventilated barn throughout the experimental period. Prior to the start of the trial, lambs were adapted from a pelleted lamb creep feed (S.C. Feeds, Stone, UK) containing 18% protein, 10% fibre, 4.5% oil and 8.5% ash during a seven day adaptation period. The creep feed was gradually replaced with the basal diet (see Table 3.1).

3.2.2. Diet formulation

The raw materials used for this study were chosen because of their published low Cu and Mo contents (MAFF, 1992). Based on this information, diets formulated were predicted to provide a Cu content of <5 mg kg^{-1} DM. The basal diet was formulated to provide the metabolisable energy (ME) and metabolisable protein (MP) requirements of a 25 kg female lamb to grow at 200 g day^{-1} (AFRC, 1993) and had a predicted effective rumen degradable protein (ERDP) of 107.42 kg^{-1} DM and an fermentable metabolisable energy (FME) of 9.99 MJ kg^{-1} DM.

Lambs were allocated by live weight to one of four dietary treatment groups which were:

Treatment one: Basal diet (Control)

Treatment two: Basal diet + 500 mg kg^{-1} DM iron and 2 g kg^{-1} DM sulphur (Fe)

Treatment three: Basal diet + 5 mg kg^{-1} DM molybdenum and 2 g kg^{-1} DM sulphur (5 Mo)

Treatment four: Basal diet + 10 mg kg^{-1} molybdenum DM and 2 g kg^{-1} DM sulphur (10 Mo)

54

Feed samples were obtained from separate tote bags once weekly. All feed samples were analysed for dry matter, ash, crude protein, ether extract, neutral detergent fibre and neutral cellulase gamanase digestibility as described in sections 2.1.1. to 2.1.6 respectively. The raw material and chemical composition of the basal diet is presented in Table 3.1. Additional mineral inclusion per treatment diet is presented in Table 3.2.

Table 3.1: Diet formulation and analysed chemical composition of the basal diet (DM)

Ingredient (g kg^{-1})	Basal diet
Straw pellets (NaOH treated)	350
Whole barley	247
Rapeseed meal	153
Citrus pulp	100
Soyabean meal	50
Molasses	60
Mineral/vitamin premix[1]	40
Total	1000
Dry matter (g kg^{-1} DM)	880.6
Metabolisable energy (MJ kg^{-1} DM)	10.6*
Crude protein (g kg^{-1} DM)	148.7
Neutral detergent fibre (g kg^{-1}DM)	381.2
Ether extract (g kg^{-1}DM)	13.6
Ash (g kg^{-1}DM)	89.9
NCDG (g kg^{-1}DM)	733.7

*Predicted values from equations given in AFRC (1993).
[1]Mineral premix – see Table 3.2

Table 3.2. Additional mineral inclusion for the control, iron and molybdenum treatment diets (kg ton^{-1})

	Treatment diet			
Ingredient	Control	Fe	5 Mo	10 Mo
Ammonium molybdate (kg)	-	-	0.008	0.016
Iron sulphate (kg)	-	2.15	-	-
Ammonium sulphate (kg)	-	6.06	7.078	7.078
Urea (kg)[1]	3.2	0.46	-	-
Sand (kg)	6.8	1.33	2.914	2.906
Mineral premix (kg)[2]	20	20	20	20
Salt (kg)	5	5	5	5
Ammonium chloride (kg)	5	5	5	5
Total (kg)	40	40	40	40

[1]Urea (Trouw Nutrition, Northwich, Cheshire, UK)

[2] Mineral Premix (20 kg ton^{-1}) (Roche, Basel, Switzerland) containing 500,000 IU/kg Vit A, 100,000 IU kg^{-1} Vit D3, 7,500 mg kg^{-1} Vit E, 15 mg kg^{-1} selenium, 32.3 % calcium, 3.9 % sodium and 2.04 % phosphorus.

The mineral composition of the basal diet was determined by ICP-ES (see section 2.3) and is presented in Table 3.3. Molybdenum was supplemented as reagent grade ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4 H_2O$), iron was supplemented as reagent grade iron sulphate ($FeSO_4 \cdot 7H_2O$) and sulphur was supplemented as reagent grade ammonium sulphate ($(NH_4)_2SO_4$) (all BDH Laboratory Supplies, Poole, Dorset, UK). The nitrogen content of the mineral mix was balanced with urea (Trouw Nutrition, Northwich, Cheshire, UK) and sand was used as an inert material to balance mass.

Table 3.3 Analysed mineral composition of the basal diet

Element	Quantity
Calcium (g kg^{-1})	8.60
Phosphorus (g kg^{-1})	3.00
Sulphur (g kg^{-1})	2.20
Magnesium (mg kg^{-1})	1.60
Copper (mg kg^{-1})	5.47
Molybdenum (mg kg^{-1})	1.29
Iron (mg kg^{-1})	167.35
Zinc (mg kg^{-1})	97.44
Cobalt (mg kg^{-1})	0.52
Selenium (mg kg^{-1})	0.0065
Manganese (mg kg^{-1})	98.12

Fresh water was offered *ad libitum* and the copper content of the water was 38 μg/l, iron <20 μg/l and no detectable molybdenum (Meridian Science, South Staffordshire Water PLC, UK).

3.2.3. Experimental Routine

All lambs were fed at 08:00 and 15:00 daily into individual feed boxes to prevent mineral contamination. Feed intake was rationed in order to achieve a growth rate of 200 g day^{-1}. All feed was weighed into individual feed buckets using metric scales and calibrated using metric standard weights (F.J. Thornton and Co. Ltd., Wolverhampton, UK). Food refusals were weighed back twice weekly to determine individual lamb food intakes. The quantity of the feed offered was recalculated weekly according to the live weight of the animal taken on the day of live weight determination (see section 3.2.3.1.).

3.2.3.1. Live weight determination

All lambs were weighed once weekly on Mondays at 11:00 using the Standard Operating Procedure as described in section 2.3. Daily liveweight gain (DLWG) was calculated using regression analysis.

3.2.3.2. Blood collection and analysis

Blood samples were collected once weekly on Tuesdays at 10:00 as described in section 2.2. Fresh whole blood was used to assess haematocrit (section 2.2.1.) and the remaining blood prepared as described in section 2.2. and stored at -20°C for subsequent analysis. Frozen samples were thoroughly defrosted and whole blood was subsequently analysed for superoxide dismutase activity (SOD) (section 2.2.5.) and haemoglobin (Hb) (section 2.2.2.), plasma analysed for plasma copper concentration (Pl-Cu) (section 2.2.6.), and serum analysed for ceruloplasmin activity (CP) (section 2.2.4.) and amine oxidase activity (AMOX) (section 2.2.7.). The intra assay coefficient of variation for respective standards were: SOD 4.83 %, Pl-Cu 3.42 %, CP 10.15 %, Hb 0.63 % and AMOX 6.65 %.

3.2.4. Liver copper concentration

All forty-eight lambs were sent to a commercial slaughterhouse on day 73 of the trial. All lambs including the six initial slaughter lambs killed on day 0 were killed using the same slaughter procedure in which they were stunned with a captive bolt pistol and killed by exsanguination. All livers were collected immediately and were frozen at -20°C. Liver Cu concentration was determined using the method from the Official Methods of Analysis (AOAC, 1990) using an atomic absorption spectrophotometer (section 2.2.8.). The intra assay coefficient of variation for the liver sample standard was 2.53 %.

3.2.5. Statistical analysis

Statistical analysis was performed using Genstat version 5.0. All haematology and enzyme parameters were analysed by analysis of variance (ANOVA) and repeated measures (where appropriate) using a completely randomized design. Daily liveweight gain (DLWG) was calculated by regression analysis and analysed by ANOVA. In all graphs, error bars represent 2x s.e.d. Differences between treatment means was calculated using a protected least significant difference (LSD) (Snedecor and Cochran, 1989).

3.3. Results

3.3.1. Growth rate and performance characteristics

There was no significant effect of 500 mg kg^{-1} Fe or 5 or 10 mg kg^{-1} Mo on lamb live weight at any weekly time point (Fig. 3.1.). There was no significant effect of 500 mg kg^{-1} Fe or 5 or 10 mg kg^{-1} Mo on total gain, daily live weight gain (DLWG), daily dry matter intake (DMI), total dry matter intake (DMI) or food conversion efficiency (FCE) (Table 3.4.). The DLWG for all treatment groups was higher than the predicted DLWG of 200g day^{-1} as formulated by AFRC (1993).

Table 3.4 *Effect of molybdenum or iron supplementation on growth rate and performance of growing lambs (kg)*

	Treatment					
	Control	Fe	5 Mo	10 Mo	s.e.d.	Significance
Initial liveweight	25.9	26.5	25.1	26.3	1.22	NS
Slaughter weight	45.2	45.3	44.7	46.2	1.77	NS
Total Gain	19.3	18.8	19.6	19.9	1.20	NS
DLWG (kg day^{-1})	0.28	0.26	0.27	0.28	0.016	NS
Daily DMI	1.54	1.54	1.50	1.59	0.059	NS
Total DMI	110.8	110.7	108.0	114.4	4.23	NS
FCE *	0.17	0.17	0.18	0.17	0.550	NS

*FCE calculated as Total Gain (kg) divided by Total DMI (kg)
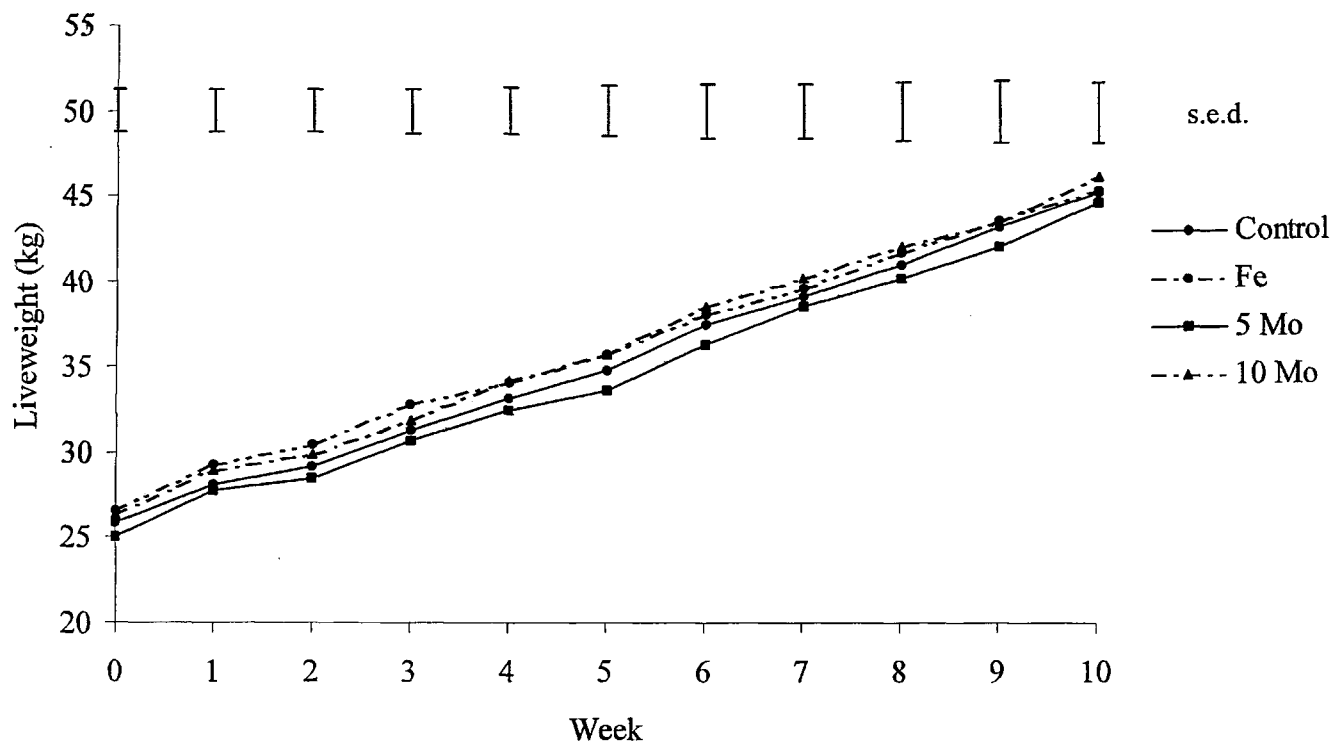
Figure 3.1.   *Effect of molybdenum or iron supplementation on live weight of growing lambs*

3.3.2. Haematocrit

All haematocrit (Hc) levels were considered to be within the normal reference ranges for sheep (22-40%) (see Appendix 1). There was no significant effect of 500 mg kg^{-1} Fe or 5 or 10 mg kg^{-1} Mo on Hc (%) of growing lambs over the experimental period (Table 3.5). There was a trend for Hc (%) to decrease in all experimental treatment groups over the ten week experimental period. Repeated measures analysis indicated that there was an effect (P<0.001) of time on Hc (%) as seen by a gradual decrease in all treatments. There was no effect of treatment or treatment x time interaction on Hc (%) of growing lambs supplemented with 500 mg kg^{-1} Fe or 5 or 10 mg kg^{-1} Mo.

Table 3.5.   *Effect of molybdenum or iron supplementation on haematocrit (%) of growing lambs*

	Treatment					
	Control	Fe	5 Mo	10 Mo	s.e.d.	Significance
Week						
0	38.0	37.6	38.3	37.2	1.06	NS
1	37.1	36.3	36.7	35.8	0.97	NS
2	34.9	34.6	34.4	34.5	0.95	NS
3	35.8	34.6	34.1	34.8	0.88	NS
4	36.9	35.4	35.3	34.5	0.87	NS
5	35.0	34.8	34.1	33.9	0.94	NS
6	35.2	34.5	34.1	34.3	0.91	NS
7	34.4	34.4	34.0	33.5	0.72	NS
8	34.3	34.3	33.9	33.8	0.86	NS
9	34.2	34.1	33.5	33.6	0.74	NS
10	34.2	33.0	33.3	32.8	0.70	NS

Repeated Measures Analysis: Treatment effect   P = NS

Time effect   P = <0.001

Time X Treatment effect   P = NS

3.3.3. Haemoglobin concentration

All haemoglobin (Hb) concentrations were considered to be within the normal reference ranges for sheep (8-16 g/dl) (see Appendix 1). There was no significant effect of 500 mg kg^{-1} Fe or 5 or 10 mg kg^{-1} Mo on Hb concentration in growing lambs except at week 9 (Table 3.6.). At week 9, the lambs that received the control dietary treatment had a significantly higher Hb concentration compared to all other dietary treatments. Repeated measures analysis indicated that there was an effect (P<0.001) of time due to fluctuations in Hb concentration by all treatment groups but no significant treatment effect or treatment x time interaction on Hb concentration.

Table 3.6. Effect of molybdenum or iron supplementation on haemoglobin concentration (g/dl) in growing lambs

| Week | Treatment | | | | | |
	Control	Fe	5 Mo	10 Mo	s.e.d.	Significance
0	11.1	11.8	11.6	10.7	0.62	NS
1	12.6	12.6	12.5	12.3	0.39	NS
2	11.1	11.4	11.2	11.4	0.56	NS
3	12.2	12.3	12.1	12.2	0.40	NS
4	13.2	12.4	12.7	12.4	0.32	NS
5	11.8	11.0	10.4	10.7	0.67	NS
6	12.3	12.3	11.9	12.1	0.55	NS
7	12.4	12.5	12.5	12.0	0.32	NS
8	11.5	11.6	11.0	11.4	0.45	NS
9	13.2[b]	12.3[a]	12.5[a]	11.9[a]	0.32	***
10	12.2	11.8	11.8	11.7	0.28	NS

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, *** <0.001

Repeated Measures Analysis:	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

3.3.4. Plasma Copper concentration

At week 1 and week 2, the lambs that received the 10 mg kg^{-1} Mo dietary treatment had higher (P<0.01) Pl-Cu concentrations compared with the lambs that had received the control or 500 mg kg^{-1} Fe dietary treatments (Fig 3.2.). At week 1, the lambs that received the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.01) Pl-Cu concentrations compared with the lambs fed the control or 500 mg kg^{-1} Fe dietary treatments. At week 2, lambs that received the 500 mg kg^{-1} Fe dietary treatment had lower Pl-Cu concentrations compared to the lambs that received the 5 mg kg^{-1} Mo dietary treatment. From weeks 3 to 10 inclusive, the lambs that received the 10 mg kg^{-1} Mo dietary treatment had higher (P<0.001) Pl-Cu concentrations compared to the lambs that received the 500 mg kg^{-1} Fe dietary treatment. From weeks 1 to 10 inclusive (albeit week 5), lambs fed the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.001) Pl-Cu concentrations compared with the lambs fed the 500 mg kg^{-1} Fe dietary treatment. There were no differences at any weekly time point in Pl-Cu concentrations of the lambs that received the 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments. The lambs that received the control treatment diet had higher (P<0.001) Pl-Cu concentrations compared with the lambs that received the 500 mg kg^{-1} Fe dietary treatment during weeks 3, 9 and 10. Pl-Cu concentrations of lambs that received the control treatment diet were lower (P<0.001) compared with the lambs fed 10 mg kg^{-1} Mo (week 4), 5 mg kg^{-1} Mo and 10 mg kg^{-1} Mo (week 6), 10 mg kg^{-1} Mo (week 7) and 10 mg kg^{-1} Mo (week 8).

Repeated measures analysis indicated that there was an (P<0.001) effect of treatment on Pl-Cu concentration due to the 10 Mo dietary treatment having the highest Pl-Cu concentration throughout the experimental period and the lowest Pl-Cu concentrations in the Fe treatment. There was no effect of time but there was an effect (P<0.05) on the time x treatment interaction as described for the treatment effect.

64
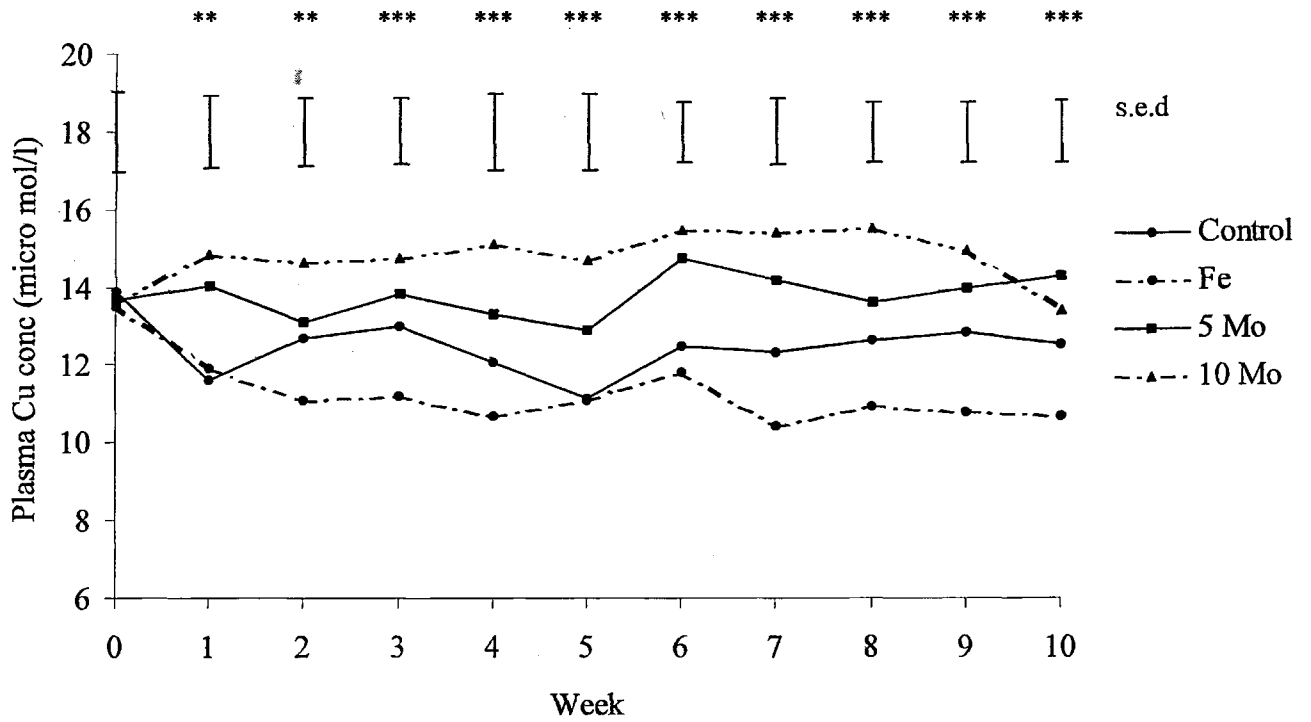
Figure 3.2. *Effect of molybdenum or iron supplementation on plasma copper concentrations of growing lambs*

Repeated Measures Analysis: Treatment effect   P = <0.001

                                Time effect   P = NS

                                Time X Treatment effect   P = <0.05

3.3.5. Ceruloplasmin Activity

At weeks 2 and 10, lambs that received the control dietary treatment had higher (P<0.01) CP activities compared with the lambs that received the 500 mg kg^{-1} Fe, 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments (Fig. 3.3.). At weeks 7, 8 and 9, lambs that received the control dietary treatment also had higher (P<0.001) CP activities compared with the lambs that received the 500 mg kg^{-1} Fe, 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments. There was no difference in mean CP activity between lambs that received 500 mg kg^{-1} Fe, 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments at any time point. Repeated measures indicated that there was an effect of dietary treatment (P<0.01) due to the control treatment having higher CP activities compared to all other dietary treatments throughout the majority of the experimental period. There was an effect of time (P<0.001) as described for the treatment interactions but no treatment x time interaction on CP activity.
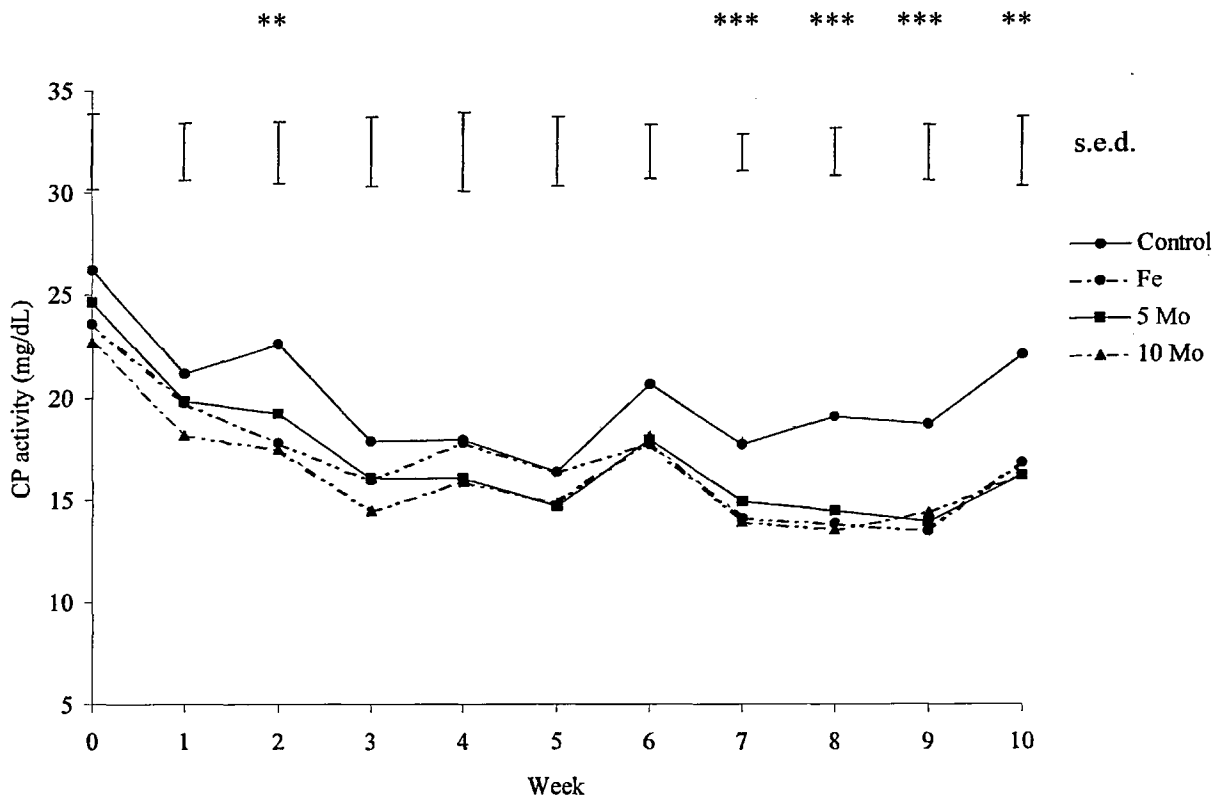
Figure 3.3. Effect of molybdenum or iron supplementation on ceruloplasmin activity of growing lambs

Repeated Measures Analysis:	Treatment effect	P = <0.01
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

3.3.6.   Ceruloplasmin to Plasma Copper Ratio (CP:Pl-Cu ratio)

From week 1 to week 10 inclusive, lambs that received the control treatment diet had higher CP:Pl-Cu ratios (P<0.001) compared to lambs that received 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo (Table 3.7). Lambs that received 10 mg kg^{-1} Mo had lower (P<0.001) CP:Pl-Cu ratios compared to lambs which received 500 mg kg^{-1} Fe from week 1 to week 10 inclusive. CP:Pl-Cu ratios were higher in the control lambs compared to the lambs that received 500 mg kg^{-1} Fe at weeks 6, 8, 9 and 10. Ratios of <1 (see Table 3.7) were calculated for the lambs given an additional 10 mg kg^{-1} Mo during weeks 7 to 9, indicative of a serious TM problem. The ratio was also beneficial in identifying significant differences in the CP:Pl-Cu ratio between lambs fed either 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo from weeks 2 to 10 inclusive.

Table 3.7.   Effect of molybdenum or iron supplementation on a ceruloplasmin to plasma copper ratio (CP:Pl-Cu) of growing lambs

Week	Treatment					
	Control	Fe	5 Mo	10 Mo	s.e.d.	Significance
0	1.92	1.75	1.82	1.67	0.099	NS
1	1.92^b	1.66^{bc}	1.41^{ac}	1.28^a	0.131	***
2	1.78^b	1.68^b	1.45^c	1.23^a	0.106	***
3	1.37^b	1.30^{bc}	1.16^c	0.98^a	0.072	***
4	1.48^b	1.46^b	1.10^c	1.04^{ac}	0.073	***
5	1.50^b	1.45^b	1.14^c	1.03^{ac}	0.108	***
6	1.66^c	1.49^b	1.22^a	1.16^a	0.063	***
7	1.44^c	1.35^c	1.06^b	0.92^a	0.057	***
8	1.50^d	1.26^c	1.06^b	0.89^a	0.047	***
9	1.45^c	1.24^b	1.00^a	0.94^a	0.068	***
10	1.73^c	1.54^b	1.14^{ab}	1.20^a	0.088	***

^{a,b} Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, *** <0.001

3.3.7. Superoxide Dismutase Activity

There was no significant effect of 500 mg kg^{-1} Fe, 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo on superoxide dismutase (SOD) activity of growing lambs over the ten week trial period, except at week nine (Fig 3.4.). At week nine, the lambs fed the control diet had higher ($P<0.05$) SOD activities compared with lambs that received the 5 mg kg^{-1} Mo and 10 mg kg^{-1} Mo dietary treatments. Lambs fed the 500 mg kg^{-1} Fe dietary treatment had higher ($P<0.01$) mean SOD activities compared with the lambs that received the 10 mg kg^{-1} Mo dietary treatment. Repeated measures analysis indicated that there was no effect of dietary treatment and no effect of a time x treatment interaction. There was an effect of time ($P<0.001$) as seen by a decrease in SOD activity amongst all treatment groups by the end of the experimental period (Fig. 3.4).
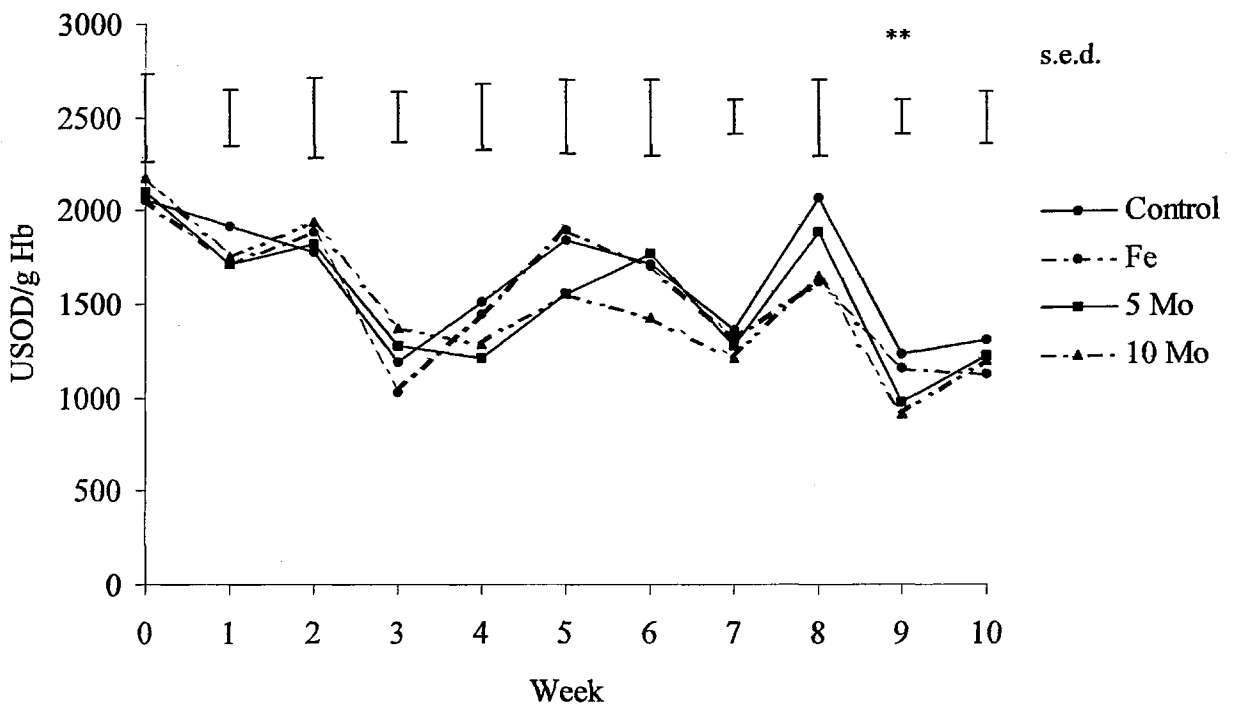
Figure 3.4.   Effect of molybdenum or iron supplementation on superoxide dismutase activity of growing lambs

Repeated Measures Analysis:	Treatment effect	$P = NS$
	Time effect	$P = <0.001$
	Time X Treatment effect	$P = NS$

3.3.8. Serum Amine Oxidase activity

At weeks 2, 3, 5 and 6 inclusive, lambs that received the control treatment diet had higher (P<0.05) AMOX activity compared with lambs that received the 10 mg kg^{-1} Mo dietary treatment (Fig. 3.5.). At week 6, lambs that received the control treatment diet also had higher (P<0.05) AMOX activity compared with lambs that received 500 mg kg^{-1} Fe. There were no differences in AMOX activity between the lambs supplemented with 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo at any time point. Repeated measures analysis indicated that there was an effect (P<0.001) of time, with a gradual increase in AMOX activity in all treatment groups. There was no effect of treatment or time x treatment interaction on AMOX activity.
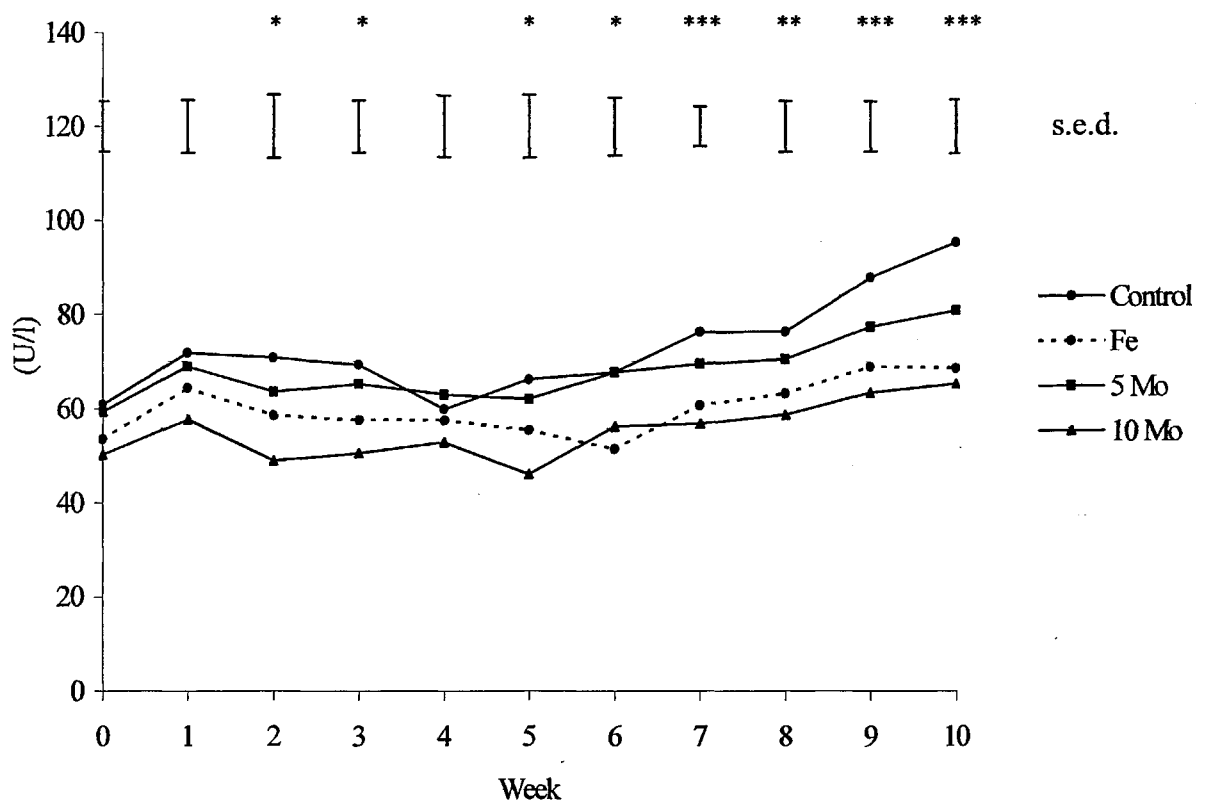
Figure 3.5. *Effect of molybdenum or iron supplementation on serum amine oxidase activity of growing lambs*

Repeated Measures Analysis:	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

3.3.9. Liver copper concentration

Mean liver Cu concentration in the initial slaughter group lambs was 216.7 mg kg^{-1} DM. Lambs that received the control treatment diet for the ten week trial period had higher (P<0.001) liver Cu concentrations compared with the pre-treatment group (278.1 mg kg^{-1} DM). After the ten week trial period, lambs that received the control treatment diet had higher (P<0.001) liver Cu concentrations compared with lambs that received 500 mg kg^{-1} Fe, 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo (Fig. 3.6.). Liver Cu concentrations in the lambs that received 500 mg kg^{-1} Fe dietary treatment had higher (P<0.001) liver Cu concentrations compared with the lambs that received 10 mg kg^{-1} Mo. There were no differences in liver copper concentrations between the lambs that received 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo.
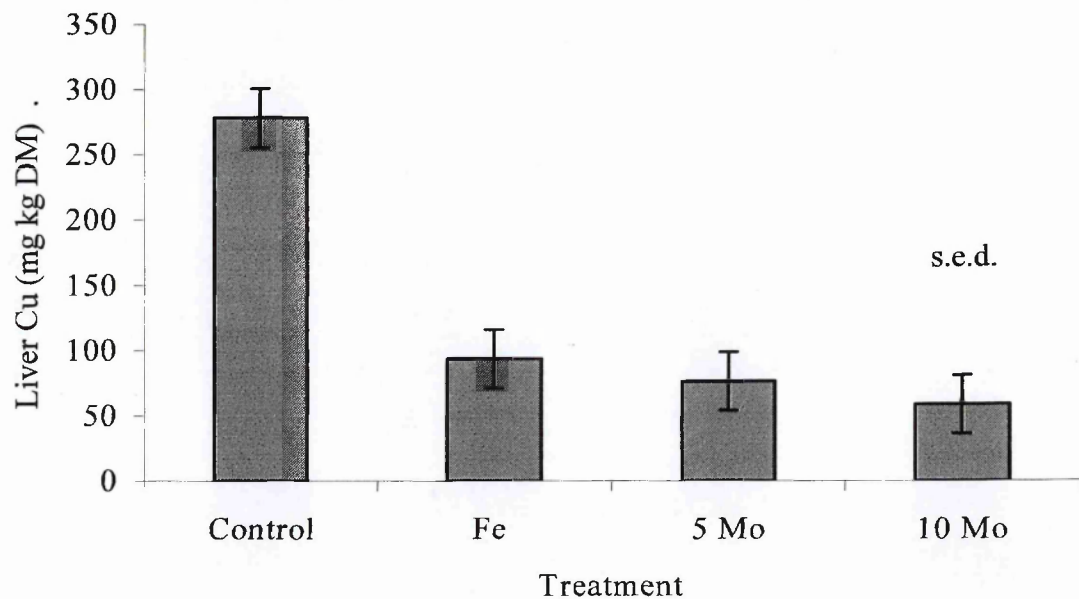
Figure 3.6.   Effect of molybdenum or iron supplementation on liver copper concentration of growing lambs

3.4. Discussion

Chemical composition of the experimental diets were very similar to the values predicted during diet formulation (Table 3.1.) and were within normal ranges, as given in MAFF (1992). Copper content of the basal diet was 5.47 mg kg^{-1} which was marginally higher than the predicted Cu requirement during diet formulation of <5 mg kg^{-1}. The Mo content in the basal diet (1.29 mg kg^{-1} DM Mo) was lower than the normal recommendations of 2-3 mg kg^{-1} DM Mo (ARC, 1980) and selenium content of the basal diet was also very low.

Dietary Mo or Fe had no significant effect on lamb growth rate or performance characteristics. These results are in accordance with the work of Phillippo et al. (1987a) and Bremner et al. (1987). In this study, DLWG was higher than the formulated 200 g day^{-1} as predicted from AFRC (1993) although DMI was almost identical to the predicted intake of 1.5 kg day^{-1}. The high DLWG for growing lambs in this trial may have been due to a breed effect that is not accounted for in prediction equations provided by AFRC (1993).

Dietary Mo produced no clinical symptoms in this study. These findings confirm those of Wentink et al. (1999) who suggested that low levels of Cu within the blood and tissues may not produce any clinical symptoms. Work by other authors found that calves receiving diets containing 800 mg kg^{-1} and 500 mg kg^{-1} Fe DM respectively were found to show no clinical symptoms (Humphries et al., 1983; Phillippo et al., 1987a) similar to lambs in this study. Therefore clinical symptoms may not be dependent on Cu status alone.

Low Hb or Hc in ruminant animals is often associated with anaemia due to mineral deficiencies, imbalances or if a Cu deficiency has been severe or prolonged (Suttle and Angus, 1978; Gooneratne et al., 1989a). The inclusion of Mo or Fe in this study had no significant effect on haematocrit (Hc) or haemoglobin (Hb) in accordance with Galbraith et

71

al. (1997) in goats. Although the 5 mg kg^{-1} Mo dietary supplement was considered to be relatively high for growing lambs (ARC, 1980), they may not have been severe or prolonged enough to alter Hb concentration within a ten week trial period.

The antagonistic effects of Mo and Fe on Cu metabolism in growing lambs were clearly identified in this study. Studies by other authors found dietary Mo to reduce Pl-Cu concentrations (Humphries *et al.,* 1983; Phillippo *et al.,* 1987a; Xin *et al.,* 1991). However, dietary Mo has also been extensively reported to markedly increase Pl-Cu concentrations (Dick, 1953; Suttle and Field 1968; Bremner and Young, 1978), in accordance with results obtained in this study. It is also known that the liver is the main storage organ of Cu (Underwood, 1977) and that CP is produced at a constitutive rate within the liver (Gitlin *et al.,* 1992). Therefore, factors should be taken into consideration when determining alterations in Pl-Cu and liver Cu concentration. The increase in Pl-Cu concentration as observed in this study may have been due to the presence TM complexes in the blood as described by Suttle (1980). TM complexes have been reported to circulate in the blood stream and due to their high affinity for Cu, act systemically, sequestering Cu from the Cu enzymes and tissues. Copper bound to TM within the blood is biologically unavailable, but may result in misleadingly high Pl-Cu concentrations due to the Cu bound to the TM (Suttle, 1980). These misleadingly high Pl-Cu concentrations are often mis-interpreted as being within 'normal' reference ranges for ruminant animals as suggested by Claypool *et al.* (1975). To maintain Cu homeostasis within the body, as Cu reserves become depleted in the blood and tissues, more Cu is released from the liver, to regulate blood Cu concentrations (Dick, 1956). Although the liver acts as the main storage organ of Cu, it may become depleted due to dietary Mo (Van Ryssen and Stielau, 1981). Dick (1956), Marcilese *et al.* (1969) and Van Niekerk and Van Niekerk (1989a) all found that liver Cu concentrations were drastically decreased in ewes or rams fed Mo alone or Mo in combination with S.

This study confirms that dietary Mo reduced liver Cu concentrations. Lambs that received the dietary Mo treatment diets had significantly reduced liver Cu concentrations, although Pl-Cu concentrations increased, compared to the lambs that received the control diet. Interestingly, Pl-Cu concentrations decreased in the dietary Fe treatment groups at a very early stage of the study and remained lower than all other treatment groups throughout the trial period. Although Cu absorption is known to be inhibited by dietary Fe (Humphries *et al.*, 1983; Bremner *et al.*, 1987; Phillippo *et al.*, 1987a), normally, the liver would have an adequate short term storage of Cu. It has been suggested that high Fe intakes may reduce Cu absorption by insoluble Fe x Cu compounds (Suttle and Peter, 1985). If dietary Fe becomes complexed to Cu, then the Mo content of the basal diet, although relatively low, may also be absorbed and have an additional inhibitory effect on Cu metabolism. Inhibition of Cu absorption may also be dependent on the S content in the rumen (Bremner *et al.*, 1987). The reduction in liver Cu concentration in Fe supplemented animals in this study is in accordance with steers (Humphries *et al.*, 1983), calves (Humphries *et al.*, 1985) and heifers (Phillippo *et al.*, 1987a). Phillippo *et al.* (1987a) found that liver Cu concentrations were significantly reduced in heifers fed diets containing the same dietary Fe concentrations as in this study compared to control fed heifers.

Mechanisms for the release of Cu from the liver either arise from ceruloplasmin (CP) which accounts for 90-95% of circulating plasma Cu (Terada *et al.*, 1995) or are due to the systemic effects of TM which increase biliary excretion and deplete hepatic Cu stores (Gooneratne *et al.*, 1989b; Symonds and Ke, 1989). The results from this study clearly indicate that dietary Mo significantly reduced CP activity in growing lambs and that this decrease in CP activity was mirrored by a decrease in liver Cu concentration. Ovine CP is known to only have a half-life of approximately 2-3 days (Linder, 1991). Therefore, TM may be complexing Cu with such a strong affinity that Cu in the CP protein is not being

recycled back to the liver and broken down, and as such the half-life of CP may be altered, resulting in a reduction in liver Cu concentration.

Another possible explanation for the observed reduction in the active CP enzyme may have been due to the direct inhibition by TM. Suttle (1980) suggested that TM complexes in the rumen may cause a reduction in Cu availability. Although CP is known to be constitutively expressed within the liver (Gitlin et al., 1992), work by Chidambaram et al. (1984) reported that TM produced irreversible inactivation in vitro of the CP enzyme, although later work by Lannon and Mason (1986) found that inactivation of CP was reversible in vivo in Jersey steers. Therefore, TM may directly affect the expression rate of the CP protein or cause an increase in the release of the apo-form of the protein, rather than the holo-protein. The increased release of the apo-CP protein may produce a pool of circulating CP that is less biologically active than the holo-protein. Similar to Pl-Cu, CP levels which appear to be within the "normal" range for ruminants should also be viewed with caution as it is not known if the CP measured was the apo or holo form. CP can also act as an acute phase protein and high levels may not be indicative of a 'normal' Cu status but may also be a reflection of stress, infection or parasitism (Mulryan and Mason, 1992).

Work by Ogra et al. (1999) using Wistar rats proposed a different mechanism by which CP may be altered by TM. Intraperitoneal injections of tetrathiomolybdate (TTM) significantly reduced the activity of CP in serum due to a reduced Cu incorporation into the Cu chaperones in the liver rather than a direct removal of Cu from the CP enzyme in the serum. Recent work has identified an intracellular Cu chaperone, Sheep Atx1 Homologue (SAH), which has been found to be important in delivering Cu in the hepatocytes from the cytoplasm to ATP7B in the transgolgi network where Cu is incorporated into CP (Lockhart and Mercer, 2000). This important finding has shown that Cu incorporation into CP is not a passive process but involves specific Cu chaperones within hepatocytes. The homologue of ATP7B in the gram-positive bacterium Enterococcus hirae has also been found to be

74

inhibited by TTM *in vitro* (Bissig *et al.*, 2001). Therefore, a more precise understanding of where TM may be altering CP expression in ruminant animals is required. It is not known whether a reduction in CP activity may be attributable to defects in the Cu chaperone protein SAH as a consequence of TM involvement or if a reduction in CP activity is due to the control of gene expression.

The CP:Pl-Cu ratio as proposed by Mackenzie *et al.* (1997) clearly identified animals that had reduced CP:Pl-Cu ratios due to dietary Mo. Ratios obtained for lambs that received the 10 mg kg^{-1} Mo dietary treatment were significantly lower compared to control fed lambs. According to ratios proposed by Mackenzie *et al.* (1997), ratios obtained in this study for the lambs that received either of the Mo dietary treatments were indicative of TM problems (ratio of 1.0 – 1.5) although from week 7 to 9 inclusive, lambs that received the 10 mg kg^{-1} Mo dietary treatment had CP:Pl-Cu ratios indicative of serious TM problems (<1.0). The mechanism by which high dietary Fe reduces Cu absorption is not fully understood but the CP:Pl-Cu ratio identified the lambs that received Fe as having a reduced CP:Pl-Cu ratio, possibly due to a similar systemic mechanism to that of TM. This requires further investigation.

Superoxide dismutase has often been used as an indicator for diagnosing Cu deficiency due to the long lifespan of erythrocytes (Kerr, 2002) and the slow decrease in enzyme activity (Suttle and McMurray, 1983) compared to the sensitivity and decreases observed in CP. Results from this study indicate that there was no significant time x treatment interaction on SOD activity, which may have been due to the relatively short experimental period of this trial. SOD appears to be more tolerant of dietary Mo or Fe compared to CP or Pl-Cu, and hence, a decrease in SOD activity may only be indicative of a severe or prolonged Cu deficiency (Paynter, 1987). Ogra *et al.* (1999) found that intraperitoneal injections of TTM in rats reduced Cu in SOD due to the removal of Cu from the Cu in the

chaperones within the liver rather than direct removal of Cu from the SOD enzyme. The same biological scenario may be true of SOD in the liver of ruminant animals.

Amine oxidase activity has shown to be reduced in experimentally induced primary Cu deficiency in cattle (Mills *et al.* 1976). Although Cu is contained within the active site of amine oxidase, it is rarely used as an indicator of Cu deficiency. Kelleher and Mason (1986) showed that AMOX activity of CP was inhibited by sodium TTM *in vitro* but few studies have shown reductions in AMOX activity *in vivo* due to dietary Mo. In this present study, AMOX activity was significantly lower in the lambs that received dietary Mo compared to control lambs. This may be due to the systemic effect of TM or a reduction in the ability of a Cu chaperone to incorporate Cu into the active site as suggested for SOD and CP by Ogra *et al.* (1999). Lambs supplemented with Fe in this study also had significantly lower AMOX activity compared to the control lambs.

3.5. Conclusion

The results from this study indicate that dietary Mo may significantly alter Cu metabolism in growing lambs. Therefore, a reduction in Cu for Cu dependent enzymes is not as a result of low Cu intakes but is rather a result of elevated intakes of dietary Mo. The effects of dietary Mo and the subsequent systemic effects on Cu parameters within the blood and tissues may be explained by the formation of insoluble TM complexes within the rumen, although these were not measured in this study. The increase in Pl-Cu concentration confirms that Pl-Cu concentration is not a useful independent indicator of Cu status in ruminant animals. Ceruloplasmin and AMOX activity were affected by dietary Mo but the CP:Pl-Cu ratio was a more useful indicator in distinguishing those animals which may have been affected by TM. The significant reductions in liver Cu concentration in lambs that received dietary Mo may have also been attributable to TM and the resultant homeostatic mechanism of the liver in releasing Cu. Alterations in Cu concentrations in the blood and tissues due to dietary Mo or Fe did not produce any clinical symptoms. In addition, the results from this study did not confirm if the differences observed in Fe supplemented lambs occurred within the digestive tract or due to a systemic effect.

To conclude, Mo was a more potent antagonist on Cu metabolism than Fe. Mineral levels and the time period chosen in this study did not affect physical performance, although more research needs to be undertaken to elucidate both cellular aspects and physiological responses of dietary Mo or Fe on Cu metabolism in ruminant animals.

Chapter 4

The effect of dietary molybdenum or iron on innate and adaptive immune responses of growing lambs

4.1. Introduction

An increased susceptibility to disease has previously been observed in grazing lambs following pasture improvement (Woolliams *et al.*, 1986a). Woolliams *et al.* (1986a) found a higher rate of mortality in lambs which had previously grazed improved pastures due to a range of bacterial infections following post-mortem examination compared to Cu supplemented lambs grazing on the same pasture. Therefore, this would suggest that Cu is an essential nutrient required for an effective immune system in ruminant animals.

In chapter 3, dietary Mo was found to lower CP activity in association with an increase in Pl-Cu, possibly due to the presence of absorbed TM having a systemic effect within the blood and tissues. Therefore, the systemic effect of absorbed TM may also alter immune function. Dietary Mo has previously been shown to alter immune responses in ruminant animals by reducing cell mediated immune responses to phytohemagglutinin (PHA) in steers (Ward *et al.*, 1993), reduce antibody responses to porcine erythrocytes (IgG and IgM) in calves (Gengelbach and Spears, 1998) and reducing phagocytic ability of neutrophils to kill ingested *Candida albicans* (Boyne and Arthur, 1986) or *Staphlococcus aureus* (Xin *et al.*, 1991) in heifers and steers respectively.

In chapter 3, intakes of dietary Fe were also found to alter Cu metabolism. Dietary Fe has previously been found to alter immune responses in ruminant animals. Fe-supplemented calves have been found to have lower secondary antibody responses to porcine erythrocytes compared to control calves (Ward *et al.* 1997). Lymphocyte proliferation tests using PHA have shown a lower secondary *in vitro* viability in Fe-supplemented calves

compared to control calves (Ward *et al.*, 1997). Iron supplemented cattle and calves were also been found to have reduced SOD activities compared to the respective control groups after twenty eight weeks of dietary treatment (Boyne and Arthur, 1986) in addition to lower leucocyte counts in calves (Gengelbach *et al.*, 1997). The phagocytic ability of neutrophils to kill *Candida albicans in vitro* has also been found to be lower in Fe-supplemented calves compared with control calves (Boyne and Arthur, 1986).

As shown in chapter 3, the Cu antagonists Mo and Fe produced differential effects on the biochemical assessments of Cu status in growing lambs after ten weeks on trial which may indicate a systemic and/or gut effect of absorbed dietary Mo within the blood and tissues. Therefore, the aim of this study was to investigate the effects of dietary Mo and Fe on the innate and adaptive immune response in growing lambs.

4.2. Materials and methods

4.2.1. Experimental Design and Animals

Twenty-four Charollais X Friesland lambs (four male and four female per treatment) of approximately 14 weeks of age were used in this study. Lambs had an initial mean live weight of 31.0 kg (s.e.d. 1.75) and were randomly allocated to one of three dietary treatment groups based on live weight (see section 4.2.2.). All lambs were housed on straw bedding and divided into their three treatment groups by metal partitionings (approx. $7m^2$ area) in an open-ventilated barn for a period of 12 weeks. Prior to the start of the trial, all lambs were adapted from an *ad libitum* pelleted lamb creep feed (S.C. Feeds, Stone, UK) based on 18% protein, 10% fibre, 4% oil and 8.5% ash onto the basal control diet (see section 4.2.2.) over a fourteen day adaptation period.

4.2.2. Diet formulation

The raw materials used for this study were chosen because of their published low Cu and Mo contents (MAFF, 1992). Based on this information, the diets formulated were predicted to provide a Cu content of <5 mg kg^{-1} DM. The basal diet was formulated to provide the metabolisable energy (ME) and metabolisable protein (MP) requirements of a 25 kg lamb growing at 200 g day^{-1} when fed *ad libitum* (AFRC, 1993) and had a predicted ERDP of 94.55 g kg^{-1} DM and an FME of 9.92 MJ kg^{-1} DM.

Lambs were allocated by live weight to one of three dietary treatment groups which were:

Treatment one: Basal diet (Control)

Treatment two: Basal diet + 500 mg kg^{-1} DM iron and 2 g kg^{-1} DM sulphur (Fe)

Treatment three: Basal diet + 5 mg kg^{-1} DM molybdenum and 2 g kg^{-1} DM sulphur (5 Mo)

Feed samples were obtained from separate tote bags once weekly. All feed samples were analysed for dry matter, ash, crude protein, ether extract, neutral detergent fibre and neutral cellulase gamanase digestibility (NCGD) as described in sections 2.1.1. to 2.1.6 respectively. The raw material and chemical composition of the basal diet is presented in Table 4.1. Additional mineral inclusion per treatment diet is presented in Table 4.2.

Table 4.1.   Diet composition and analysed chemical composition of the basal diet (DM)

Ingredient (g kg^{-1})	Basal Diet
Straw pellets (NaOH treated)	400
Rolled barley	300
Rapeseed meal	200
Molasses	65
Mineral premix[1]	35
Total	1000
Dry Matter (g/kg DM)	860.9
Metabolisable energy (MJ kg^{-1} DM)	10.5*
Crude protein (g kg^{-1} DM)	137.6
Neutral detergent fibre (g kg^{-1} DM)	464.1
Ether extract (g kg^{-1} DM)	14.3
Ash (g kg^{-1} DM)	97.8
NCGD (g kg^{-1} DM)	722.9

* Predicted values from equations given in AFRC (1993) (see appendix 2)
[1]Mineral Premix – see Table 4.2.

Table 4.2. Additional mineral inclusion for the control, iron and molybdenum treatment diets (kg^{-1} ton)

	Treatment diet		
Ingredient	Control	Fe	5 Mo
Ammonium molybdate (kg)	-	-	0.008
Iron sulphate (kg)	-	2.15	-
Ammonium sulphate (kg)	-	6.06	7.078
Urea (kg)[1]	3.2	0.46	-
Sand (kg)	6.8	1.33	2.914
Mineral premix[2]	25	25	25
Total (kg)	35	35	35

[1] Urea (Trouw Nutrition, Northwich, Cheshire, UK)

[2] Mineral premix (25 kg^{-1} ton) commercial lamb vitamin/mineral premix (Frank Wright Feeds Ltd, Ashbourne, Derbyshire, UK) containing 500,000 IU Vit A, 100,000 IU Vit D3, 7500 mg kg^{-1} Vit. E, 15 mg kg^{-1} selenium, 32.3 % calcium, 3.9 % sodium and 2.04 % phosphorus.

The mineral composition of the basal diet was determined by ICP-ES (see section 2.3) and is presented in Table 4.3. Molybdenum was supplemented as reagent grade ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$, iron was supplemented as reagent grade iron sulphate $(FeSO_4.7H_2O)$ and additional sulphur was supplemented as reagent grade ammonium sulphate $(NH_4)_2SO_4$ (all BDH Laboratory Supplies, Poole, Dorset, UK). The nitrogen content of the mineral mix was balanced with urea (Trouw Nutrition, Northwich, Cheshire,UK) and sand was used as an inert material to balance mass.

Table 4.3. Analysed mineral composition of basal diet

Element	Content
Calcium (g kg^{-1})	10.60
Phosphorus (g kg^{-1})	4.50
Sulphur (g kg^{-1})	3.00
Magnesium (mg kg^{-1})	2.00
Copper (mg kg^{-1})	8.59
Molybdenum (mg kg^{-1})	0.88
Iron (mg kg^{-1})	197.18
Zinc (mg kg^{-1})	67.90
Cobalt (mg kg^{-1})	1.65
Selenium (mg kg^{-1})	0.076
Manganese (mg kg^{-1})	86.51

Fresh water was offered *ad libitum* and the copper content of the water was 35 µg/l and no detectable molybdenum (Dee Valley Group Laboratory, Rhostyllen, Wrexham, UK).

4.2.3. Experimental Routine

All lambs were routinely checked twice daily. Feed was replenished daily into feed troughs at 08:00 to maintain *ad libitum* feed requirements.

4.2.3.1. Live weight determination

All lambs were weighed once weekly on Mondays at 11:00 using the Standard Operating Procedure as described in section 2.3., calibrated using metric standard weights (F.J. Thornton and Co. Ltd., Wolverhampton, UK.). Daily live weight gain was calculated using regression analysis.

4.2.3.2. Blood collection and analysis

Blood samples were collected from all lambs on Tuesdays at 11:00 as described in section 2.2. Fresh whole blood was used to assess haematocrit (Hc) (section 2.2.1.) and the remaining blood prepared as described in section 2.2 and stored at -20°C for subsequent analysis. Frozen samples were thoroughly defrosted and whole blood was subsequently analysed for superoxide dismutase activity (SOD) (section 2.2.5.) and haemoglobin (Hb) (section 2.2.3.), plasma analysed for plasma copper concentration (Pl-Cu) (section 2.2.6.), and serum analysed for ceruloplasmin activity (CP) (section 2.2.4.), haptoglobin (section 4.2.4.) and anti-KLH IgG and IgM (section 4.2.7.4.). The intra assay coefficient of variation for respective standards were: SOD 5.64 %, Pl-Cu 6.6 %, Hb 3.08 % and CP 7.36 %. During weeks 6, 9 and 12 of the trial, additional blood was taken from 12 selected lambs (four per treatment) on two consecutive days (all 24 lambs) into lithium heparin vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) for the lymphocyte transformation test (LTT) (see section 4.2.5.). During weeks 8, 10 and 12, blood was also collected into lithium heparin vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) for the neutrophil function test (see section 4.2.6.).

4.2.4. Haptoglobin

Haptoglobin was analysed using a Phase™ Acute Phase Assay kit (Tridelta Development Ltd., County Kildare, UK) based on an adapted method of Eckersall et al. (1999). The assay was performed in a Cobas Mira Plus autoanalyser (ABX Diagnostics, Shefford, Bedfordshire, UK) at 37°C. The principle of the assay was based on the binding of haptoglobin to haemoglobin present in the specimen that preserves the peroxidase activity of haemoglobin at a low pH. Preservation of the peroxidase activity of haemoglobin was proportional to the amount of haptoglobin present in the specimen. Serum samples were defrosted to room temperature and prepared according to the Haptoglobin Kit method. The intra assay coefficient of variation for the standard was <0.1 %.

4.2.5. Lymphocyte Transformation Test (LTT)

The Lymphocyte Transformation Test (LTT) assay used isolated blood mononuclear cells isolated by density gradient centrifugation. All steps were performed in a laminar flow cabinet to maintain aseptic conditons. A tetrazolium salt, MTT (3(4,5-dimethylthiazoyl-2-yl) 2,5 diphenyltetrazolium bromide) (Sigma Aldrich, Gillingham, Poole, Dorset, UK) was used to study transformed lymphocytes using an adapted method of Mosmann (1983).

Heparinised whole blood was diluted 1:1 with phosphate buffered saline (PBS) (Hudson and Hay, 1989) (Appendix 2). Subsequently, 6 ml of diluted blood was layered onto 6 ml of 62.5% Percoll and centrifuged at 400*g* for 40 minutes at 4°C. Cells were carefully collected from the interface and then washed three times in PBS by repeated re-suspension and centrifugation. Viable mononuclear cells were counted using an improved Neubauer haemocytometer (Weber Scientific International Ltd., Middlesex, UK) using 0.2 % Nigrosine solution (BDH Laboratory Supplies, Poole, Dorset, UK) and diluted to a concentration of 0.5×10^6 mononuclear cells (MNC) per ml with tissue culture medium (TCM). The TCM contained 5 % heat inactivated neonatal calf serum (NCS), 20 mM sodium bicarbonate, 2 mM L-glutamine, 2 µg/ml Amphoteracin B, and 100 µg/ml Gentamycin Sulphate made up in RPMI 1640 (all products obtained from Sigma Aldrich, Gillingham, Poole, Dorset, UK).

To each duplicate well, 180 µl of cell suspension was added to a 96-well, round-bottomed sterile micro-titre plate (Becton Dickinson, Poole, Dorset, UK). Each cell population was stimulated by the addition of 20 µl of RPMI 1640 solution (Sigma Aldrich, Gillingham, Poole, Dorset, UK) containing either 5 µg/ml Concanavalin A (ConA), 5 µg/ml Pokeweed Mitogen (PWM) or 25 µg/ml Keyhole Limpet Haemocyanin (KLH) giving a final optimal concentration of 20 µg/ml. Control wells contained 180 µl cell suspension and 20 µl RPMI prepared in duplicate wells. The same batch of each mitogen was used throughout the experiment. All plates were incubated at 37°C in a humidified 5% CO_2 incubator (Sanyo Gallenkamp PLC, Leicester, UK) for 48 hours. To each culture well, 20 µl of 5 mg/ml 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, Gillingham, Poole, Dorset, UK) in PBS was added and incubated at 37°C in 5 % CO_2 for a further 4 hours. Plates were then centrifuged at $300g$ for 5 minutes and the supernatant aspirated off and discarded. To each well, 200 μl of dimethyl sulfoxide (DMSO) (BDH Laboratory Supplies, Poole, Dorset, UK) working solution (1:10 1M HCL to DMSO) was added. All plates were left to stand for 15 minutes. All plates were read on ELISA reader (Benchmark microplate reader, Bio-Rad, Hemel Hempstead, Herts, UK) at a wavelength of 570 nm (OD_{570}) and a reference wavelength of 630 nm (OD_{630}).

4.2.6. Neutrophil Function Test

Two different neutrophil function tests were performed for this study. Assay one was based on the ability of peripheral blood neutrophils to attach and phagocytose yellow fluorescent carboxylate beads (phagocytosis) similar to the method of Oda and Maeda (1986). Assay two was based on internally generated reactive oxidants from neutrophils (Flaminio *et al.* 2000).

4.2.6.1. Neutrophil Isolation

Blood samples were taken from all lambs as described in section 2.2. at weeks 8, 10 and 12. One ml of fresh whole blood was obtained from a lithium heparin vacutainer tube (see section 2.2.), added to 20 ml of Lysis buffer (4.13g NH_4Cl, 0.5g $KHCO_3$ and 0.017 g Na_4EDTA in 0.5 L distilled water) and incubated at 37°C for 10 minutes. Cell suspensions were centrifuged for 7 minutes at $2500g$ and the remaining pellet washed three times by repeated centrifugation and resuspension in 1 ml PBS in a micro centrifuge tube. The pellet was then centrifuged for a further 5 minutes at $7000g$. The remaining cell suspension was re-suspended in 500 μl PBS and left at 4°C until required.

4.2.6.2. Assay one – carboxylate beads

Three μl of a 1:10 dilution of yellow carboxylate beads (Molecular Probes Inc., Eugene, Oregon, USA) was pipetted into 100 μl of cell suspension (see section 4.2.6.1.). To a comparative control sample, 3 μl of PBS was added. All cells were incubated for 30 minutes at 37°C and were subsequently washed twice by centrifugation and resuspension using 1 ml of 10mM EDTA in PBS. 50 μl of cell-fix (Becton Dickinson, Oxford, UK) was added to the 500 μl of cell suspension and left at 4°C until required. Neutrophil activity was assessed using a FACScan (see section 4.2.6.4.).

4.2.6.3. Assay two - Internally generated reactive oxidants

Into a micro-centrifuge tube, D-632 (Sigma Aldrich, Gillingham, Poole, Dorset, UK) was dissolved in DMSO to give a 20 μM solution. A further 1:2 dilution was then prepared using PBS. To a test sample, 10 μl of the 1:2 D-632 was added to 100 μl of cell suspension (see section 4.2.6.1.). To a comparative control sample, 10 μl of PBS was added to a 100 μl cell suspension. All cells were incubated for 5 minutes at 37°C. To the test sample, 1 μl of 0.1 mg/ml Phorbol Myristate Acetate (Sigma Aldrich, Gillingham, Poole, Dorset, UK) (PMA) diluted 1:2 with PBS was added to the cell suspension and 1 μl PBS was added to the control sample. All cells were incubated for 20 minutes at 37°C. Into each micro-centrifuge tube, 50 μl of cell-fix (Becton Dickinson, Oxford, UK) was added to the 500 μl of cell suspension and left at 4°C until required. Neutrophil activity was assessed using a FACScan (see section 4.2.6.4.).

4.2.6.4. Flow cytometric evaluation

Flow cytometric evaluation was performed on all neutrophil populations using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Neutrophil populations were discriminated on the basis of forward scatter (FSC) versus side scatter (SSC) cytograms of peripheral mixed blood leukocyte cell populations

(PMBL) as described by McTaggart *et al.* (2001). Fluorescence excitation was at 495 nm and emission at 525 nm. All collected data was analysed using WinMDI Version 2.5 programme and the results expressed as the mean fluorescence intensity (MFI).

4.2.7. Assessment of humoral immune responses in lambs

The lambs' humoral immune responses to a novel antigen were determined by the procedure of Pollock *et al.* (1991).

4.2.7.1. Preparation of Keyhole Limpet Haemocyanin (KLH)

Keyhole Limpet Haemocyanin (KLH) (Calibochem, Novabiochem, UK) was dissolved in PBS, pH 7.2 (Hudson and Hay, 1989) to give a 1 mg/ml solution and sterilised through a 0.2 μm sterile acrodisc syringe filter (Pall Gelman Corporation, Michigan, USA) into sterile 30 ml universal tubes (Sarstedt, Boston Road, Leicester, UK). The solution was precipitated on alum by the method described by Hudson and Hay (1989). In brief, 0.45 ml of filter sterilised 1M sodium bicarbonate (BDH Laboratory Supplies, Poole, Dorset, UK) was added to each ml of KLH solution followed by the slow addition of 1.0 ml filter-sterilised 0.2M aluminium potassium sulphate (BDH Laboratory Supplies, Poole, Dorset, UK). The suspension was allowed to stand at room temperature for 15 minutes before being centrifuged at 300*g* for 15 minutes. The precipitate was washed three times by centrifugation and resuspension with sterile PBS before being resuspended to the original volume in PBS. This suspension was considered to contain 1 mg/ml of KLH.

4.2.7.2. Immunisation protocol

All lambs were immunised with 1 ml of 1 mg/ml KLH injected subcutaneously using a 20 gauge (1.5 cm) needle (Becton Dickinson Vacutainer Systems, Plymouth, UK) and a 2 ml syringe at a constant site above the ribs. Each site of injection was swabbed with 70 %

ethanol on cotton wool prior to immunisation. The same procedure was performed for both immunisation timepoints at week 4 and 8.

4.2.7.3. Optimisation assay for anti-IgG and anti-IgM using ammonium sulphate precipitation of ovine serum

Prior to analysis of test samples, the optimum concentration of monoclonal antibodies (Mab) was assessed. Into a glass centrifuge tube, 4 ml of ovine serum was added. Whilst being continuously stirred using a magnetic stirrer, 2.66 ml of saturated ammonium sulphate (SAS) (BDH Laboratory Supplies, Poole, Dorset, UK) (450 g in 500 ml distilled water) was slowly pipetted into the serum. The solution was continuously stirred for 5 minutes and then left to stand at 4°C for 15 minutes. The solution was centrifuged at 3000g for 15 minutes and the supernatant removed. The precipitate was then resuspended in 6 ml PBS and a further 4 ml of SAS was slowly added to the suspension, with continuous stirring. The solution was left for a further 20 minutes at 4°C and then centrifuged at 3000g for 15 minutes. The precipitate was then dissolved in 1 ml purite water and dialysed through a D9277 cellulose membrane (Sigma Aldrich, Gillingham, Poole, Dorset, UK) into PBS (pH 7.4). After 16 hours, the protein was pipetted from the cellulose membrane into a micro-centrifuge tube. A 1:2 dilution was prepared with PBS and the absorption of protein concentration was assessed specrophotometrically (Beckman DU640, Fullerton, California) at OD_{280nm} and OD_{260nm} using the following calculation:

$$\text{Protein concentration (mg ml}^{-1}) = (1.55 \times A_{280}) - (0.77 \times A_{260})$$

(Practical Biochemistry, 1994)

Using the above calculation, dilutions were prepared with PBS to produce a 5 µg/ml purified protein (γ-globulin). To each well of a sterile 96-well ELISA plate (Becton Dickinson, Poole, Dorset, UK), 100 µl of a 5 µg/ml of purified protein was added. All plates were incubated at 37°C for 2 hours in a humified box in a 5% CO_2 incubator (Sanyo Gallenkamp PLC, Leicester, UK). All plates were washed three times with PBS and 100 µl

of 0.05% Tween (0.5 ml in 1 litre PBS) was added to each well. Into separate micro centrifuge tubes, 1:100 dilutions of mouse anti-ovine-IgG and mouse anti-ovine-IgM antibodies (Serotec Ltd., Kidlington, Oxford, UK) were prepared in PBS/Tween (Sigma Aldrich, Gillingham, Kent, UK). To each well in column one of the ELISA plate, 100 μl dilutions of mouse anti-ovine-IgG and mouse anti-ovine-IgM were added and serial dilutions were prepared across the plate (1:100 to 1:204,800). ELISA plates were incubated for one hour at 37°C and then subsequently washed three times in PBS/Tween. To all wells, 100 μl of 1:30,000 Sigma goat-anti-mouse alkaline phosphatase conjugate was added. All ELISA plates were incubated for one hour at 37°C and then subsequently washed three times in PBS/Tween. To all wells, 100 μl of Sigma phosphatase substrate (Sigma Aldrich, Gillingham, Kent, UK) (1mg/ml in glycine buffer at pH 10.4) was added. Colour intensity was allowed to develop in the dark at room temperature and optical density was assessed using an ELISA reader (Benchmark microplate reader, Bio-Rad, Hemel Hempstead, Herts, UK) at a wavelength of OD_{405nm}. Optimal IgG and IgM was estimated using a standard curve (Fig. 4.1.) and dilutions of 1:1600 were determined to be optimal for both IgG and IgM respectively.
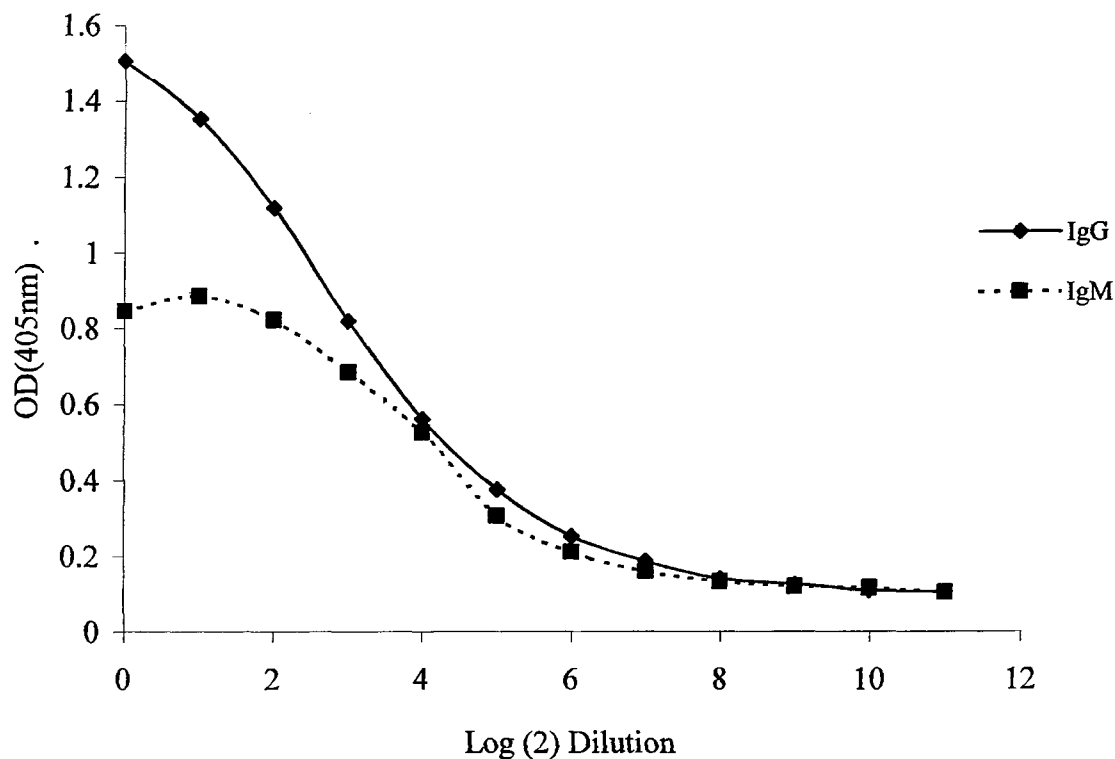
Fig. 4.1. Optimisation assay for IgG and IgM monoclonal antibodies using ovine serum

4.2.7.4. Preparation of Anti-KLH ELISA

One hundred μl of 10 μg/ml KLH in PBS was added to each well of a 96-well ELISA plate (Becton Dickinson Labware, New Jersey, USA). All plates were incubated at 37°C for one hour to allow the KLH to bind and then left overnight at 4°C. Plates were washed three times with PBS to remove any excess KLH. Uncoated plastic was blocked with 150 μl of 0.1% Bovine Serum Albumin (BSA) (Sigma Aldrich, Gillingham, Poole, Dorset, UK) in PBS and incubated for a further two hours at 37°C. Plates were then washed three times with PBS containing 0.05% Tween 20 (BDH Laboratory Supplies, Poole, Dorset, UK) (PBS/T) and thoroughly dried. To duplicate wells, 100 μl of 1:50 diluted serum in PBS/T was added and all plates incubated at 37°C for one hour. Plates were then washed a further three times in PBS/T. To each appropriate well, 100 μl of 1:1600 dilutions in PBS monoclonal antibody (anti-ovine-IgG or anti-ovine-IgM) (Serotec, Kidlington, Oxford, UK) were added. Plates were then incubated for one hour at 37°C and then washed three

91

times in PBS/T. Plates were thoroughly dried and 100 µl of 1:30,000 Sigma anti-mouse alkaline phosphatase conjugate (Sigma Aldrich, Gillingham, Poole, Dorset, UK) was added to each well. Plates were then incubated for a further one hour at 37°C and washed three times in PBS/T. To each well, 100 µl of Sigma S104 phosphatase substrate (1 mg/ml in glycine buffer, pH 10.4) (Sigma Aldrich, Gillingham, Poole, Dorset, UK) was added. All plates were then left in dark conditions to allow colour to develop. Optical densities were then read on an ELISA plate reader (Benchmark microplate reader, Bio-Rad, Hemel Hempstead, Herts, UK) at a wavelength of 405nm (OD_{405}) until the internal standard had an OD of 1-1.2.

4.2.8. Liver copper determination

On day 84 (week 12), all lambs were sent to a commercial slaughterhouse and killed by electrical stunning and exsanguination. All livers were removed and immediately placed in individual clean plastic bags and frozen at -20°C. Liver trace element concentration was subsequently determined by ICP-MS (Section 2.2.9.).

4.2.9. Statistical analysis

Statistical analysis was performed using Genstat for Windows (Version 5.0). All haematology and enzyme parameters, trace element content of tissue samples, LTT, anti-KLH IgG and anti-KLH IgM responses were analysed by analysis of variance (ANOVA) and repeated measures (where appropriate) using a completely randomized design. Haptoglobin concentration was analysed by Kruskall Wallis (Snedecor and Cochran, 1989). Daily liveweight gain (DLWG) was determined by regression analysis and analysed using ANOVA. In all graphs, error bars refer to 2x s.e.d. Differences between treatments was calculated using a protected least significant difference (LSD) (Snedecor and Cochran, 1989).

4.3. Results

4.3.1. Growth rate and performance characteristics

There was no significant effect of 5 mg kg^{-1} Mo or 500 mg kg^{-1} Fe on lamb liveweight (Fig 4.2.) or daily live weight gain (DLWG) at any timepoint (Table 4.4.). The DLWG for all dietary treatment groups was very similar to the predicted DLWG of 200 g day^{-1} as formulated by AFRC (1993).

Table 4.4.   *Effect of molybdenum or iron supplementation on growth rate and performance of growing lambs (kg)*

	Treatment diet				
	Control	Fe	5 Mo	s.e.d.	Significance
Initial LW	31.0	30.7	31.3	1.75	NS
Slaughter wt	48.3	46.4	49.3	3.08	NS
DLWG (kg/day)	0.197	0.184	0.198	0.0217	NS
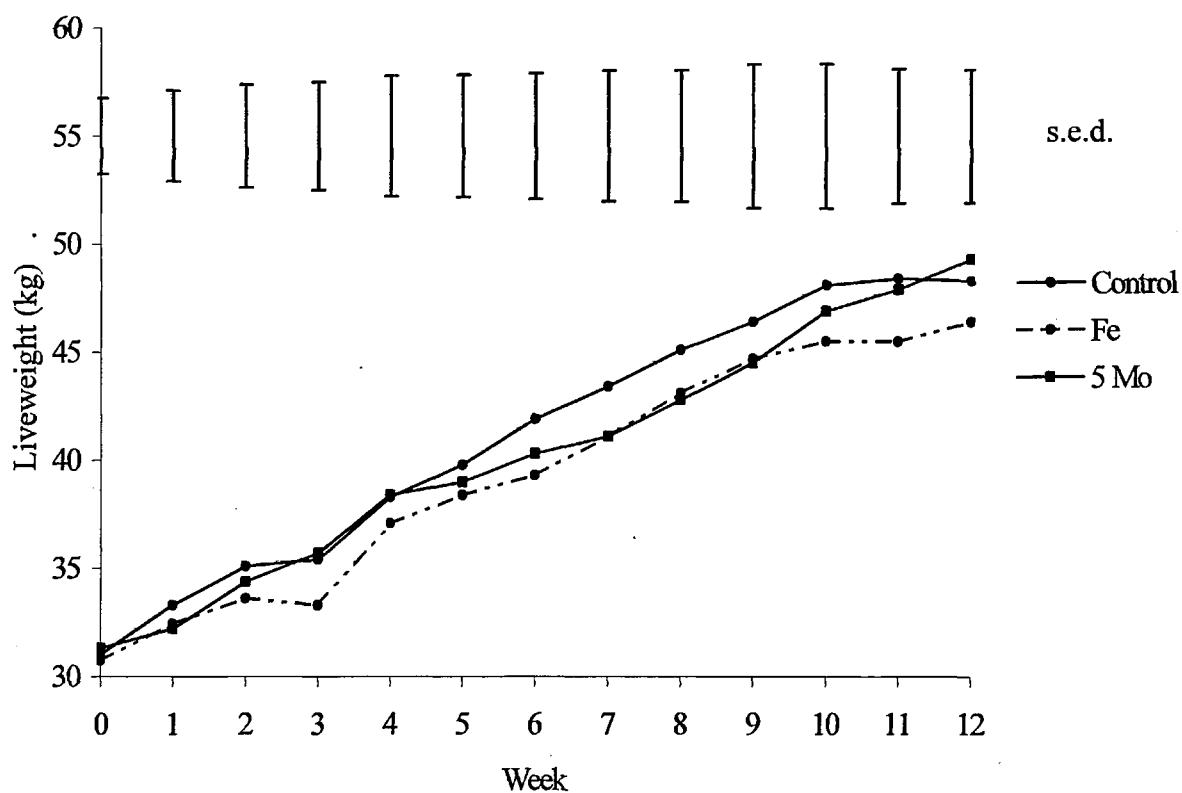
Fig 4.2.   *Effect of molybdenum or iron supplementation on live weight of growing lambs*

4.3.2. Haematocrit

There was no significant effect of 5 mg kg^{-1} Mo or 500 mg kg^{-1} Fe on Hc (%) at weeks 1 and 2 (Table 4.5.). Lambs that received the 500 mg kg^{-1} Fe dietary treatment had higher Hc (%) at week 3 (P<0.05), week 5 (P<0.01), week 6 (P<0.001) and week 7 (P<0.05) compared to lambs that received the control or 5 mg kg^{-1} Mo dietary treatments. Lambs that received the 500 mg kg^{-1} Fe dietary treatment had higher (P<0.05) Hc compared to lambs that received the control diet at week 4 and higher (P<0.05) Hc compared to lambs that received the 5 mg kg^{-1} Mo dietary treatment at week 10 respectively. There were no differences in Hc (%) between lambs that received the control or 5 mg kg^{-1} Mo dietary treatments at any timepoint. Repeated measures analysis indicated that there was no effect of treatment on Hc. There was a time x treatment interaction (P<0.01) and effect of time (P<0.001) that occurred due to a decrease in Hc in the control and 5 Mo dietary treatments over time.

Table 4.5. Effect of molybdenum or iron supplementation on haematocrit (%) of growing lambs

Week	Control	Fe	5 Mo	s.e.d.	Significance
0	35.25	33.75	36.50	1.46	NS
1	34.00	35.63	35.75	1.14	NS
2	32.38	34.50	32.75	1.01	NS
3	33.00^a	35.00^b	33.63^a	0.77	*
4	32.25^a	35.13^b	34.00^{a,b}	1.01	*
5	31.75^a	35.50^b	32.63^a	1.09	**
6	31.87^a	35.75^b	32.63^a	0.88	***
7	32.57^a	35.50^b	32.25^a	1.08	*
8	33.50	34.25	32.00	1.41	NS
9	32.88	33.50	32.25	1.58	NS
10	32.88^{a,b}	34.88^b	31.50^a	1.20	*
11	31.75	33.25	32.38	1.14	NS
12	32.63	33.71	33.43	1.27	NS

^{a,b} Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, ***<0.001

Repeated Measures Analysis: Treatment effect   P = NS

                                Time effect   P = <0.001

                                Time X Treatment effect   P = <0.01

4.3.3. Haemoglobin concentration

There was no effect of 5 mg kg^{-1} Mo or 500 mg kg^{-1} Fe on mean haemoglobin (Hb)

concentration in growing lambs at week 1 and 2 (Table 4.6.). At weeks 3, Hb concentration

of lambs that received the 500 mg kg^{-1} Fe dietary treatment was higher ($P<0.05$) compared

to the lambs that received the control dietary treatment. At week 5, mean Hb concentration

of lambs that received the 500 mg kg^{-1} Fe dietary treatment was higher ($P<0.001$)

compared to the lambs that received the control or 5 mg kg^{-1} Mo dietary treatments. Mean

Hb concentration of lambs that received the 500 mg kg^{-1} Fe dietary treatment was higher

than lambs that received either the control or 5 mg kg^{-1} Mo dietary treatments at week 6

($P<0.05$) and week 7 ($P<0.01$). At week 10, lambs that received the 500 mg kg^{-1} Fe dietary

treatment had a higher ($P<0.05$) Hb concentration compared to the lambs that received the

5 mg kg^{-1} Mo dietary treatment. There were no differences in mean Hb concentrations of

the lambs that received the control or 5 mg kg^{-1} Mo dietary treatments at any weekly time

point. Repeated measures analysis indicated that there no effect of dietary treatment on Hb

concentration, but there was an effect of time ($P<0.001$) and a time x treatment ($P<0.001$)

interaction on Hb concentration. These effects were due to the gradual decrease in Hb

concentration in all treatment groups over time.

Table 4.6. Effect of molybdenum or iron supplementation on haemoglobin concentration (g/dl) in growing lambs

		Treatment			
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	13.7	12.8	13.6	0.58	NS
1	12.0	12.5	13.4	0.57	NS
2	11.7	12.0	11.6	0.51	NS
3	13.1^a	14.4^b	$13.8^{a,b}$	0.41	*
4	11.9	12.3	12.2	0.63	NS
5	12.1^a	13.9^b	11.6^a	0.43	***
6	11.2^a	12.5^b	11.3^a	0.44	*
7	11.6^a	13.3^b	11.8^a	0.46	**
8	12.8	13.1	12.2	0.50	NS
9	12.3	12.1	12.4	0.61	NS
10	$12.5^{a,b}$	13.4^b	11.7^a	0.52	*
11	11.6	12.3	11.9	0.46	NS
12	10.9	11.4	12.0	0.43	NS

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, *** <0.001

Repeated Measures Analysis:	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.001

4.3.4. Plasma Copper (Pl-Cu) concentration

The grand mean Pl-Cu concentration for all treatment groups at week 0 was 18.4 μmol l^{-1}. Using week 0 as covariate, at week 3, lambs that received the control treatment diet had higher (P<0.05) Pl-Cu concentrations compared to lambs that received the 500 mg kg^{-1} Fe dietary treatment (Fig 4.3.) At weeks 4 and 6, lambs that received the control dietary treatment had higher (P<0.01) Pl-Cu concentrations compared to the lambs that received the 500 mg kg^{-1} Fe dietary treatment. At weeks 4 and 5, lambs that received the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.01) Pl-Cu concentrations compared to lambs that received the 500 mg kg^{-1} Fe dietary treatment. At weeks 6 and 7, lambs that received the control dietary treatment had higher (P<0.01) Pl-Cu concentrations compared to lambs that received the 500 mg kg^{-1} Fe dietary treatment. Pl-Cu concentrations were higher (P<0.01) for the lambs that received the control dietary treatment compared to the lambs that received the 5 mg kg^{-1} Mo dietary treatment at week 6. At week 7, lambs that received the 500 mg kg^{-1} Fe dietary treatment had lower Pl-Cu concentrations compared to the lambs that received the 5 mg kg^{-1} Mo dietary treatment. At weeks 11 and 12, lambs that received the control dietary treatment had higher Pl-Cu concentrations compared to the lambs that received the 500 mg kg^{-1} Fe dietary treatment. Repeated measures analysis indicated that there was a significant effect (P<0.01) of dietary treatment which was due to the decrease in Pl-Cu concentration in the Fe dietary treatment from week 1 onwards. There was a significant effect of time (P<0.001) due to the low levels of Pl-Cu in the Fe group and the increase in Pl-Cu concentration in the 5 Mo dietary treatment group from week 10 onwards. There was no time x treatment interaction on Pl-Cu concentrations in growing lambs.
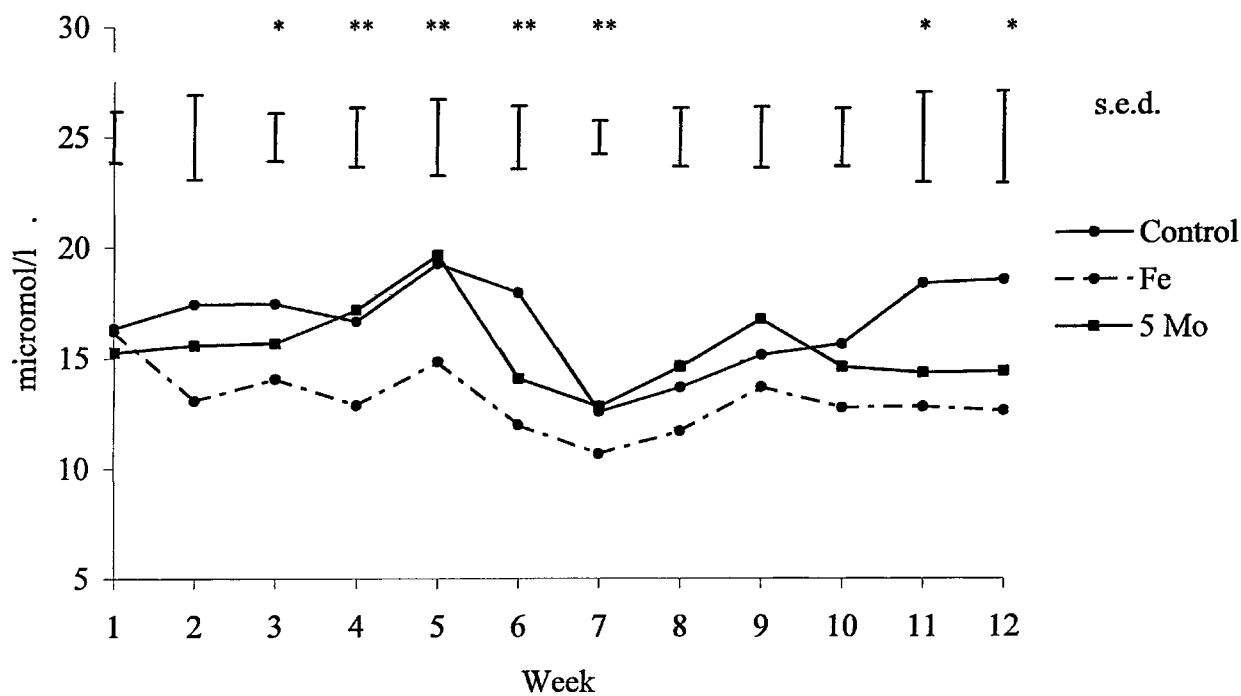
Figure 4.3.   *Effect of molybdenum or iron supplementation on plasma copper concentration of growing lambs*

Repeated Measures Analysis:	Treatment effect	P = <0.01
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

4.3.5. Ceruloplasmin activity

Mean CP activity for all treatments at week 0 was 20.68 mg/dl. Using week 0 as covariate, at week 1, lambs that received the control treatment diet had higher (P<0.001) CP activity compared to lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments (Fig. 4.4.). At weeks 2 and 4, lambs that received the control dietary treatment had higher (P<0.01) CP activity compared to the lambs that received the 500 mg kg^{-1} Fe dietary treatment. Lambs that received the control treatment diet had higher CP activity compared to the lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments at week 3 (P<0.001), week 5 (P<0.05), week 6 (P<0.01) and week 7 (P<0.001) respectively. There were no differences in mean CP activity between lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo at any time point. Repeated measures analysis indicated that there was a significant effect of treatment (P<0.001). There was a trend over the twelve week trial period for the control treatment group to have a higher mean CP level than both the 500 mg kg^{-1} Fe and 5 mg kg^{-1} Mo dietary treatment groups (Fig 4.4.). This was reflected by an effect of time (P<0.05) but no time x treatment interaction on CP activity.
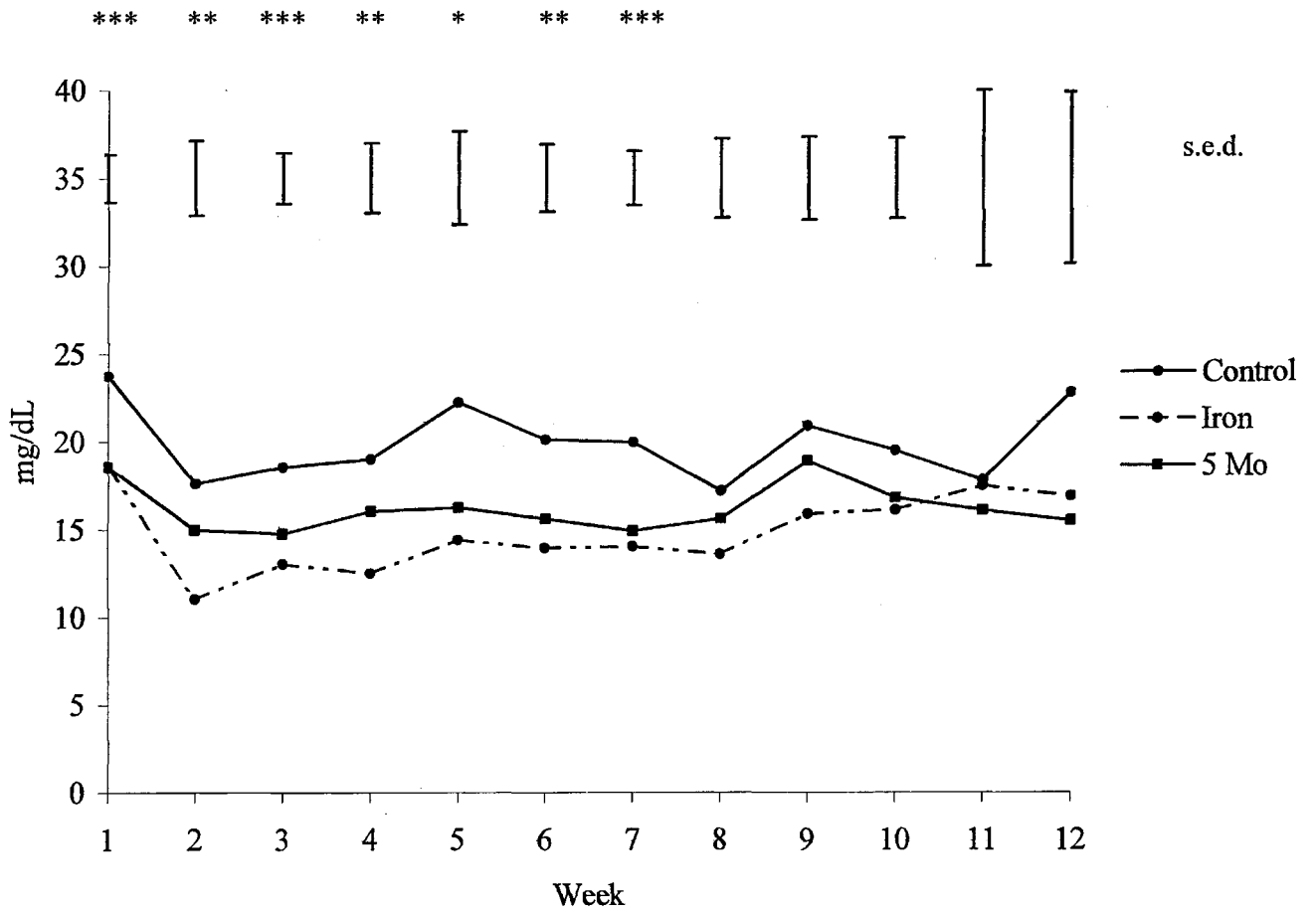
Figure 4.4. *Effect of molybdenum or iron supplementation on ceruloplasmin activity of growing lambs*

Repeated Measures Analysis:	Treatment effect	P = <0.001
	Time effect	P = <0.05
	Time X Treatment effect	P = NS

4.3.6. Ceruloplasmin to Plasma Copper Ratio (CP:Pl-Cu)

Mean CP:Pl-Cu ratio for all treatments at week 0 was 1.11. Using week 0 as covariate, at week 1, lambs that received the control diet had a higher (P<0.05) CP:Pl-Cu ratio compared to lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo (Table 4.7.). At week 3 and 4, lambs that received the 5 mg kg^{-1} Mo dietary treatment had a lower (P<0.05) CP:Pl-Cu ratio compared to lambs that received either the control or 500 mg kg^{-1} Fe dietary treatments. Lambs that received the 5 mg kg^{-1} Mo dietary treatment also had a lower CP:Pl-Cu ratio compared to lambs fed either the control or 500 mg kg^{-1} Fe dietary treatments at weeks 5 (P<0.001), week 6 (P<0.05), week 7 (P<0.001) and week 8 (P<0.05) respectively. There were no differences in the CP:Pl-Cu ratio between lambs that received the control or 500 mg kg^{-1} Fe dietary treatments at weeks 5 to 8 inclusive. At week 9, lambs that received the control dietary treatment had a higher CP:Pl-Cu ratio compared to lambs that received either the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments. There were no differences between treatments in CP:Pl-Cu ratio at weeks 10, 11 or 12. Repeated measures analysis indicated that there was an (P<0.01) effect of treatment, possibly due to the low CP:Pl-Cu ratios in the 5 Mo treatment group compared to the control and Fe groups. There was no time or time x treatment interaction on CP:Pl-Cu ratio.

Table 4.7.   *Effect of molybdenum or iron supplementation on ceruloplasmin to plasma copper ratio (CP:Pl-Cu) of growing lambs*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
1	1.34[b]	1.18[a]	1.17[a]	0.064	*
2	1.02	0.93	0.85	0.100	NS
3	1.07[b]	0.97[b]	0.86[a]	0.071	*
4	1.17[b]	1.01[b]	0.86[a]	0.101	*
5	1.17[b]	1.02[b]	0.74[a]	0.104	***
6	1.16[b]	1.21[b]	0.97[a]	0.080	*
7	1.60[b]	1.39[b]	1.06[a]	0.119	***
8	1.32[b]	1.17[b]	1.00[a]	0.120	*
9	1.36[b]	1.10[b]	1.11[a]	0.120	**
10	1.30	1.21	1.15	0.087	NS
11	0.98	1.12	1.01	0.111	NS
12	1.25	0.99	1.09	0.135	NS

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, ***<0.001

Repeated Measures Analysis: Treatment effect $P = <0.01$

Time effect $P = NS$

Time X Treatment effect $P = NS$

4.3.7. Superoxide Dismutase (SOD) activity

There was no effect of dietary treatment until week 5 on SOD activity (Fig 4.5.). Lambs that received the control dietary treatment had higher SOD activities at week 5 (P<0.05), week 7 (P<0.01), week 9 (P<0.001) and week 12 (P<0.001) respectively compared to the lambs that received the 5 mg kg^{-1} Mo dietary treatment. There was a consistent trend for the control treatment group to have higher mean SOD activity compared to lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments from week 7 onwards. Repeated measures analysis indicated that there was a effect of treatment (P<0.01) due to the SOD activity in the control group remaining higher than the Fe or 5 Mo treatment groups from week 7 to week 12. This was also reflected by an effect of time (P<0.001) but no significant time x treatment interaction.
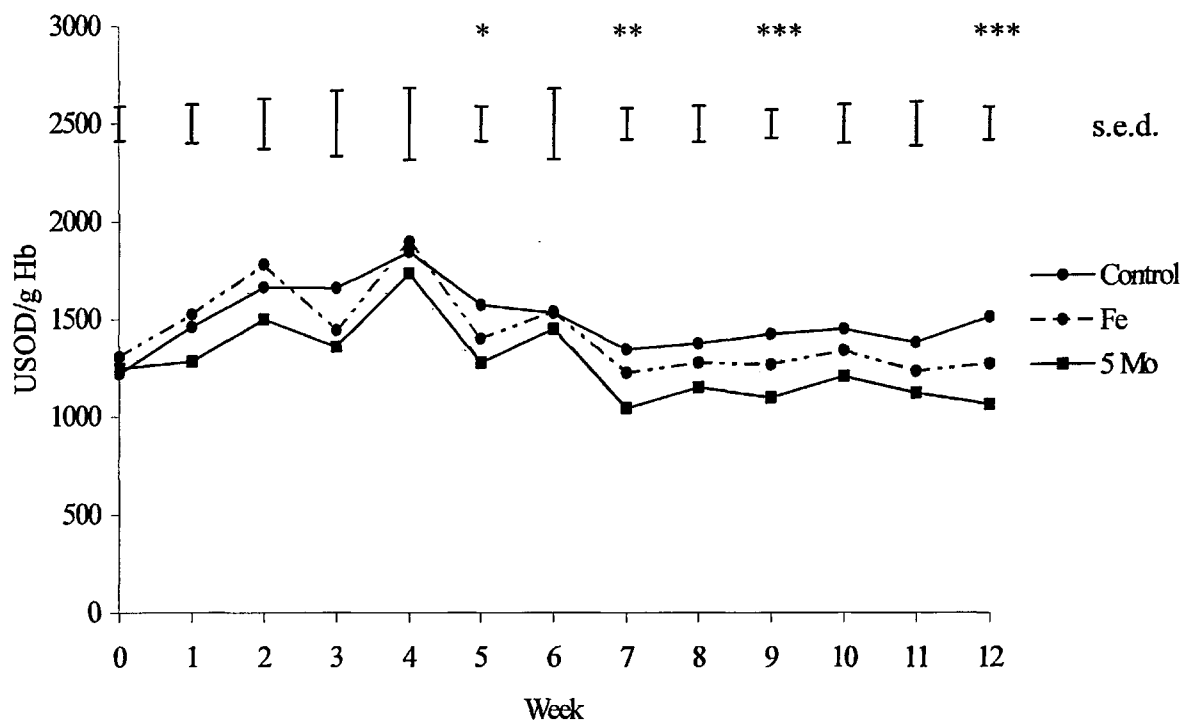
Figure 4.5. Effect of molybdenum or iron supplementation on superoxide dismutase activity of growing lambs

Repeated Measures Analysis:	Treatment effect	P = <0.01
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

4.3.8. Haptoglobin concentration

There was no effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on haptoglobin concentration in growing lambs following immunisation with KLH at weeks 4 and 8 (Table 4.8.). An increase in haptoglobin concentration occured post-immunisation at week 5, but this was not significantly different between dietary treatments.

Table 4.8. *Effect of molybdenum or iron supplementation on haptoglobin concentration (mg/ml) of growing lambs*

Week	Control	Fe	5 Mo	H-value	Significance
4	0.010	0.010	0.010	1.5010	NS
5	0.120	0.035	0.020	1.4890	NS
6	0.010	0.010	0.010	0.6612	NS
8	0.010	0.010	0.010	0.2201	NS
9	0.015	0.010	0.010	1.0620	NS
10	0.010	0.010	0.010	0.2979	NS

All figures quoted as median values

4.3.9. Lymphocyte Transformation Test (LTT)

There was no effect of dietary treatment on lymphocyte transformation tests (LTT) to Con A, PWM or KLH during weeks 6, 9 or 12 (Table 4.9.) in growing lambs.

Table 4.9.   *Effect of treatment on in vitro lymphocyte transformation test (LTT) response (OD 507nm and reference wavelength of OD 630nm) to Control, Conconavalin A (Con A), Pokweed Mitogen (PWM) and Keyhole Limpet Haemocyanin (KLH) of growing lambs*

	Treatment				
	Control	Fe	5 Mo	s.e.d.	Significance
Control					
Wk 6	1.099	1.062	1.077	0.1856	NS
Wk 9	0.497	0.690	0.727	0.1619	NS
Wk 12	1.280	1.360	1.190	0.3750	NS
Con A					
Wk 6	0.685	0.808	0.698	0.1388	NS
Wk 9	0.268	0.388	0.371	0.1344	NS
Wk 12	1.100	1.080	1.000	0.9680	NS
PWM					
Wk 6	0.765	0.735	0.953	0.1701	NS
Wk 9	0.393	0.643	0.571	0.1466	NS
Wk 12	1.250	1.190	1.080	0.3900	NS
KLH					
Wk 6	0.956	0.867	1.062	0.1792	NS
Wk 9	0.465	0.746	0.682	0.1899	NS
Wk 12	1.210	1.300	1.140	0.3890	NS

4.3.10. Neutrophil Function Test

The population of cells showing the mean fluorescence intensity (MFI) (Fig. 4.6 and Fig. 4.7) could not be identified as neutrophils as there was no known commercial antibody marker available that was specific to ovine neutrophils. CD11a/CD18 are commonly used markers but are also found on other ovine leucocyte populations (Griebel, 1998). Fig 4.6. and Fig. 4.7. are examples of cell populations obtained from the carboxylate bead test, from lamb (303) which received the control diet during week 12. Two distinct cell populations can be identified but it is not known what cell types these are. The lower cell populations are likely to be cell debris and the higher cell populations, macrophages and neutrophils. No statistical comparisons were therefore made between treatments during weeks 8, 10 and 12 in fluorescence analysis of neutrophil populations.
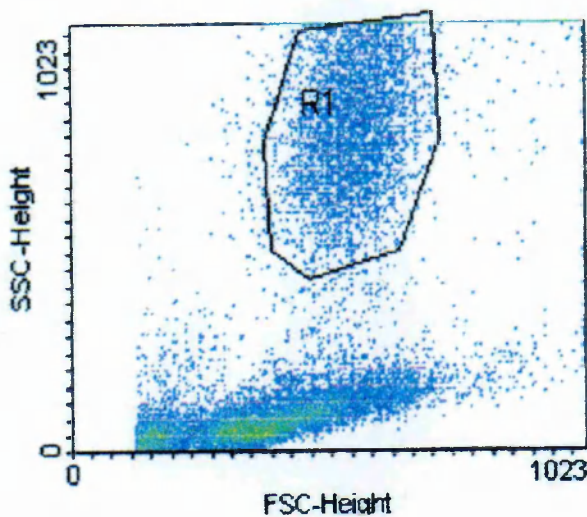
Fig. 4.6. Neutrophil function test – Carboxylate Beads (-) control ; lamb 303 (control diet), week 12
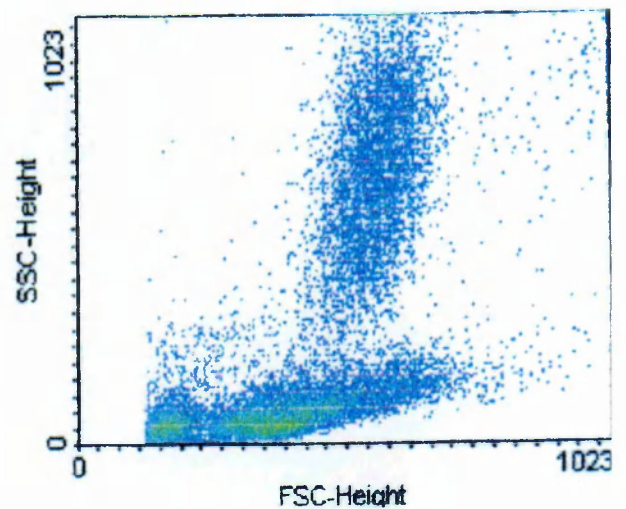
Fig. 4.7. Neutrophil function test - Carboxylate beads (+) control ; lamb 303 (control diet), week 12

4.3.11. Serum Anti-KLH IgG and IgM responses

There was no effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments on anti-KLH IgG responses (Fig 4.8) or anti-KLH IgM responses (Fig 4.9) in growing lambs at any timepoint. Although primary and secondary responses can been seen post- immunisation with KLH at weeks 4 and 8, these increases were not significantly different.
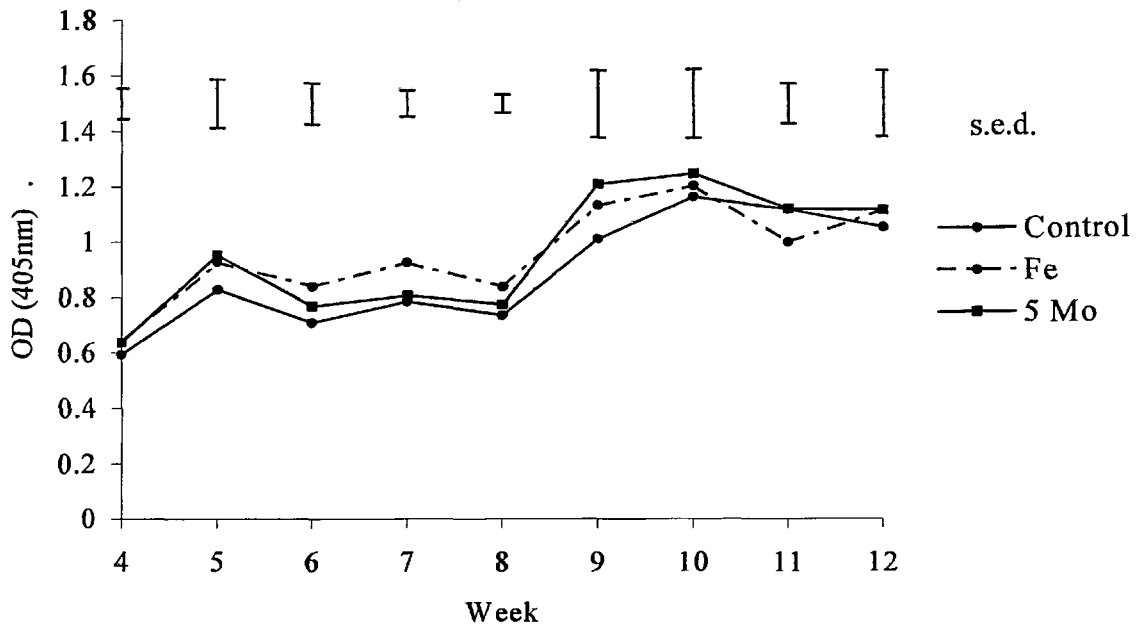
Figure 4.8. **Effect of molybdenum or iron supplementation on anti-KLH IgG response in growing lambs**
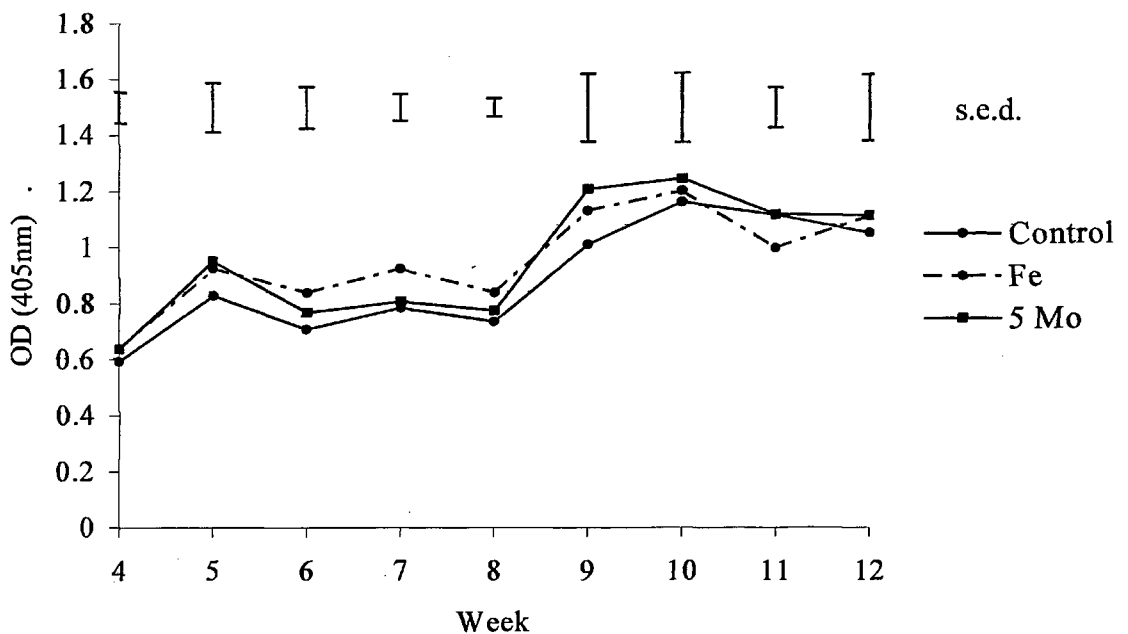
Figure 4.9. **Effect of molybdenum or iron supplementation on anti-KLH IgM response in growing lambs**

4.3.12. Trace element concentration in the liver

Liver samples were collected from all lambs at slaughter after 12 weeks on trial. Lambs that received either the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments had lower (P<0.001) liver Cu concentrations compared with lambs that received the control diet (Table 4.10.). There was no difference in liver Cu concentration between the lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments. Lambs that received the 500 mg kg^{-1} Fe dietary treatment had higher (P<0.001) liver Fe concentrations compared to lambs that received the control diet or the 5 mg kg^{-1} Mo dietary treatment. Lambs that received the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.001) liver Mo concentrations compared with the lambs that received the control or 500 mg kg^{-1} Fe dietary treatment. There were no differences in liver Mo between the control and 500 mg kg^{-1} Fe supplemented lambs. Lambs that received the control diet had higher (P<0.01) liver Zn concentrations compared with lambs that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments.

Table 4.10.   Effect of molybdenum or iron supplementation on trace element accumulation in the liver (μg g^{-1} DM $\pm$ s.e.d.) of growing lambs

	Treatment				
	Control	Fe	5 Mo	s.e.d.	Significance
Element					
Cu	251.98^b	97.33^a	98.80^a	29.901	***
Fe	109.19^a	225.09^b	86.52^a	18.830	***
Mo	1.528^a	1.682^a	2.348^b	0.1504	***
Zn	90.01^b	58.10^a	51.96^a	10.600	**

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, ***<0.001

4.4. Discussion

One level of dietary Mo (5 mg kg^{-1} Mo) was used in this trial as intakes above 1 mg kg^{-1} Mo are considered to be deleterious to Cu metabolism (Suttle, 1983b). Previous studies by other authors using the same level of dietary Mo have been found to produce significant reductions on Cu status in young cattle (Phillippo *et al.*, 1987a; Humphries *et al.*, 1983).

Chemical composition of the experimental diets were very similar to values predicted during diet formulation and were very similar to chemical analysis obtained from the diet in chapter 3. Copper content of the basal diet was found to be 8.59 mg kg^{-1} Cu which was higher than the formulated <5 mg kg^{-1} Cu. This increase in Cu content of the basal diet may have occurred due to the different feed types used in this study compared to the basal diet in chapter 3 where a Cu content of 5.47 mg kg^{-1} Cu was achieved. Feeds used in this study were chosen due to availability and because of their low published Cu values. Mineral composition of other elements contained within the basal diet were considered adequate for growing lambs (Table 4.3.) (ARC, 1980). Iron content of the basal diet in this study (197.18 mg kg^{-1} DM) was higher than that of the basal diet in chapter 3 (167.35 mg kg^{-1} DM). In contrast to chapter 3, Mo content of the basal diet in this study was lower (chapter 3: 1.29 mg kg^{-1} Mo DM; chapter 4, 0.88 mg kg^{-1} Mo DM). Therefore, these differences in basal diet composition may affect Cu metabolism, Cu status and physiological effects.

The inclusion of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo had no significant effect on lamb DLWG which is in accordance with results obtained in chapter 3 and other authors (Bremner *et al.* 1987). The results from this study showed that although Hb and Hc values for all lambs were considered to be within the normal reference ranges for sheep (Wolfensohn and Lloyd, 1998), Hc and Hb concentrations of lambs that received Fe were higher compared to lambs that received the control or 5 Mo dietary treatments. These results contradict

109

those obtained in chapter 3, where no differences in Hc or Hb concentration due to dietary treatment were found. The results from this study also contradict those of Humphries et al. (1983) who found no differences in Hc or Hb concentration in calves that received either 5 mg kg^{-1} Mo or 800 mg kg^{-1} Fe in their basal diet containing 4 mg kg^{-1} Cu. Phillippo et al. (1987a) found marginal decreases in Hb concentration in heifers that received 5 mg kg^{-1} Mo but no differences in Hc or Hb in heifers that received 500 mg kg^{-1} Fe. The Fe content of the basal diet in this study and in chapter 3 were adequate for a growing lambs (ARC, 1980) although the Fe content of the basal diet in the current study was higher than that in chapter 3.

The increased Hc and Hb concentrations in lambs in this study clearly show that dietary treatment was affecting haemopoesis. The formation of red blood cells in the bone marrow (haemopoesis) is stimulated by erythropoietin, which is secreted by the kidneys (Kerr, 2002). Results from this study showed that lambs that received the Fe dietary treatment had the lowest liver Cu concentration in association with the highest Hc and Hb concentrations during the mid-weeks of the trial. In addition, there was a trend for lambs that received the Fe dietary treatment to have the lowest CP activity and the lowest Pl-Cu concentrations. The essentiality for Cu in Hb formation in rats was first established by Hart et al. (1928). Later work by Gubler et al. (1952) showed that anaemia in swine was due to a defect of Fe metabolism, which is now associated with the CP protein. CP, also termed 'ferroxidase' is known to be essential in oxidising ferrous iron into ferric iron before incorporation into transferrin (Owen and Hazelrig, 1963). Therefore, in the absence of CP, this oxidation stage is hindered and the incorporation of iron into apo-transferrin for delivery into the site of Hb synthesis is slowed down. Mills et al. (1976) found that disturbances in Fe metabolism resulted in a decreased CP activity. The liver acts as the main storage site for blood and therefore, if the liver is being affected by dietary antagonists, this may result in an increase in Cu release from the liver for the red blood cell populations as a homeostatic mechanism to maintain Hc or Hb concentrations. Anaemia in

ruminant animals has only been reported to develop after prolonged periods of a Cu deficiency (Beck, 1941). The Fe level in the basal diet in addition to the 500 mg kg^{-1} Fe was also reflected by a significantly higher liver Fe concentration. This finding is in agreement with work by Phillippo *et al.* (1987a) in cattle.

After 12 weeks, lambs fed the control treatment diet were the only treatment group to have liver Cu concentrations considered to be within the normal range for sheep (100-500 mg kg^{-1} DM) (Underwood, 1977). In accordance with chapter 3 and previous studies by Bremner and Young (1978), Phillippo *et al.* (1987a) and Van Niekerk and Van Niekerk (1989a), liver Cu concentrations were found to be significantly reduced by the addition of Mo in the diets of the growing lambs. This decrease may be due to the systemic effects of circulating TM that may deplete blood and tissue Cu reserves. Liver Cu concentrations were also depleted in the Fe supplemented group. The mechanism by which dietary Fe alters Cu metabolism is unknown, but may be as a result of a reduction in absorption at the intestinal mucosa. Liver Mo was found to be significantly higher in lambs that received an additional 5 mg kg^{-1} Mo and liver Zn was found to be significantly higher in lambs that received the control diet although it is not known what the biological significance of this was.

Results from this study and those of chapter 3 clearly identify an effect of dietary Mo on Cu metabolism of growing lambs. It is evident from both studies that there is an inter-relationship between Pl-Cu concentration, CP activity and liver Cu concentration. In chapter 3, an increase in Pl-Cu concentration was shown to be reflected in a decrease in CP activity and also a subsequent reduction in liver Cu concentration. Work by other authors have found an increase in Pl-Cu concentrations in response to high levels of dietary Mo (Bremner and Young, 1978; Van Niekerk and Van Niekerk, 1989a; Du Plessis *et al.*, 1999a). In the current study, although increases in Pl-Cu concentration were less apparent,

111

lambs that received the Mo dietary treatment had fluctuating Pl-Cu concentrations in association with a decreasing CP activity. Price *et al.* (1987) suggested that Cu:Mo ratios of <1 in sheep would enhance the formation of TM. Therefore, as the Cu:Mo ratio in this study was >1, this may account for the lack of effect in Pl-Cu concentration. The differences in Pl-Cu concentration and CP activity between the control and Mo treatment groups were clearly identified when using the CP:Pl-Cu ratio. It remains unclear if the decrease in CP activity observed towards the latter end of the study was due to a reduction in CP expression or due to the inhibition of CP by TM. Formation of TM within the rumen (Suttle, 1974b; Dick *et al.*, 1975) causes a subsequent decrease in CP activity (Lannon and Mason, 1986) as observed in chapter 3. *In vitro* studies (Kelleher and Mason, 1986; Lannon and Mason, 1986) and *in vivo* studies (Mason *et al.*, 1980, 1982; Lannon and Mason, 1986) found that TM are potent inhibitors of both ovine and bovine CP activity. Work by Lannon and Mason (1986) found that an increase in albumin bound Cu (Pl-Cu) was not related to CP degradation and therefore, a rise in Pl-Cu concentration may be due to systemic effects of TM. Although there was no obvious increase in Pl-Cu concentration in lambs that received the Mo dietary treatment in this study, liver Cu concentration by week 12 was significantly lower than that of the control group (similar to liver Cu concentrations obtained in chapter 3). The findings from this study and from chapter 3 would suggest that due to homeostatic regulation of the liver to supply Cu to blood and tissues, the strong binding affinity of TM for Cu within the rumen, may enhance the release of Cu stored in the liver to maintain metabolic functions.

The mechanism by which dietary Fe reduces Pl-Cu concentration remains unclear. The distinct decrease in Pl-Cu concentration in Fe supplemented lambs in this study and in chapter 3, is also in accordance with other authors (Humphries *et al.*, 1983; Bremner *et al.*, 1987; Phillippo *et al.*, 1987a). Although Pl-Cu concentration returned to baseline levels (week 1) by the end of the study, Pl-Cu concentration in Fe-supplemented lambs was distinctly lower than all other treatment groups throughout the trial period. The reduction

in Pl-Cu concentration coupled with a decrease in CP activity occurred from the onset of this study, similar to results obtained in chapter 3. Results from this study, chapter 3 and work by Humphries *et al.* (1983), Bremner *et al.* (1987) and Phillippo *et al.* (1987a) have shown a relationship in a decrease in Pl-Cu concentration in association with a decrease in liver Cu concentration. It would seem plausible that a decrease in liver Cu concentration arises from similar homeostatic mechanisms within the liver as observed in Mo-supplemented lambs. It has been suggested that high Fe intakes may reduce Cu absorption by insoluble Fe x Cu compounds (Suttle and Peter, 1985). Therefore, the importance of these studies indicate that Fe can also be a potent antagonist to Cu metabolism, but the underlying mechanisms by which this occurs remain unclear.

The CP:Pl-Cu ratio was again, useful in identifying lambs receiving intakes of dietary Mo. These results are in accordance with chapter 3. Where significant differences did occur, there was a trend for the lambs that received 5 mg kg^{-1} Mo to have a significantly lower CP:Pl-Cu ratio compared to the lambs that received 500 mg kg^{-1} Fe or the control diet.

The way in which dietary Mo affects immune function has been found to be variable. This current study identified that lambs that received the 5 mg kg^{-1} Mo dietary treatment had depressed SOD activities compared to the lambs that received the control or the 500 mg kg^{-1} Fe dietary treatments from week 1 to week 12 inclusive. CuZnSOD (SOD$_1$) is an important intracellular protein required to catalyse the dismutation of the superoxide radicle ($^{\cdot}O_2^{-}$) to prevent lipid peroxidation in the cell membrane (McCord and Fridovich, 1969). The findings from this study are in accordance with Arthington *et al.* (1996) who found that Mo-supplemented heifers had decreased SOD activities after 129 days on trial and may therefore reduce the ability of SOD to catalyse the dismutation of the superoxide radical, causing cellular damage and an increased susceptibility to infection due to a reduction in neutrophil viability. Jones and Suttle (1981) suggested that the reduction in

113

SOD activity in Cu deficient sheep and cattle increased extracellular $\cdot O_2^-$ and subsequently increased the oxidative damage to normal cells due to the inability of SOD to remove these toxic by-products produced during the respiratory burst. Boyne and Arthur (1986) found that Mo or Fe severely impaired neutrophil candidacidal activity of cattle *in vitro* and also reduced SOD activity.

Another explanation for the decrease in SOD activity in this study could be due to the CuZnSOD chaperone, *CCS*. Unlike the anti-oxidant enzyme, CP, SOD activity has not been reported to be inhibited by TTM *in vitro*. Ceruloplasmin is a soluble protein in plasma, whereas SOD is cellular. Therefore, the mechanism by which TM accesses the protein may account for the differences in CP and SOD activity. CuZnSOD is dependent upon a specific Cu chaperone within the hepatocyte (*CCS*) which transports Cu in the cytoplasm to the SOD protein (Rosenzweig, 2002). Therefore, a reduction in Cu availability in addition to disturbances in incorporation of Cu into SOD may occur if *CCS* cannot effectively perform its' role as a Cu chaperone. It is possible that the formation of TM in the presence of high levels of dietary Mo may reduce the Cu which is incorporated into SOD (similar to that of CP and ATOX 1) by affecting the Cu chaperone, *CCS*, by an as yet unknown mechanism.

Due to the lack of a commercial CD_{11a}/CD_{18a} monoclonal antibody available to identify neutrophil populations on the FACscan, it was not possible to distinguish neutrophil populations from other cell types. Based on findings by other authors, it would seem plausible that a decrease in SOD activity due to dietary treatment may alter the function of neutrophils, leading to an impaired immune function.

Ceruloplasmin and SOD play critical roles in prevention of oxidative tissue damage resulting from infection and inflammation (Suttle and Jones, 1986). In this study, KLH did not induce an acute inflammatory response. The acute phase protein, haptoglobin, (a major

114

responder during an inflammatory response) or CP (a minor responder during an inflammatory response) (Connor *et al.*, 1986) remained low after immunisation with KLH. Inflammatory responses often reveal an increase in CP activity coupled with an increase in Pl-Cu concentration, as observed in Holstein steers challenged with Infectious Bovine Rhinotracheitis virus (IBRV) (Stabel *et al.* 1993). Arthington *et al.* (1996) found low CP concentrations in heifers due to dietary Mo with no increase in CP activity following BHV-1 inoculation, similar to results from this study using KLH. Therefore, the lack of an inflammatory response in this study may suggest that KLH did not provoke a significant inflammatory response compared with an inoculant such as IBRV. The adjuvant used here (alum) is not aggressive at stimulating macrophages compared to others used elsewhere by Alexander and Brewer (1995).

KLH has previously been used by other authors to activate both cellular and humoral immune responses in calves (Gasbarre, 1986 ; Pollock *et al.*, 1991). The dietary treatments in this study had no significant effect on IgG or IgM responses to KLH. Gengelbach and Spears (1998) found that calves fed a diet containing 1.1 mg kg^{-1} Cu plus an additional 5 mg kg^{-1} Mo had lower primary and secondary immune responses to porcine erythrocytes compared to calves fed a Cu supplemented diet (10 mg kg^{-1} Cu). Porcine erythrocytes use macrophages as antigen presenting cells to T helper cells whereas KLH uses B cells initially as antigen presenting cells (Roitt *et al.*, 1998) and may therefore be less sensitive to dietary Mo.

This study found no effect of dietary treatment to T or B lymphocyte blastogenic responses, similar to findings in calves by Stabel *et al.* (1993). Arthington *et al.* (1996) found no response in Mo supplemented heifers to intranasal inoculation with Bovine Herpes Virus –1 (BHV-1) in terms of lymphocyte proliferation to the mitogens Con-A or

PWM, no increase in CP activity or Pl-Cu concentration, but a decrease in SOD activity. These findings by Arthington *et al.* (1996) are similar to those found in this present study. Variation in lymphocyte isolation technique and different culture media may alter lymphocyte blastogenic responses. Pollock *et al.* (1994) found that by removing homologous serum and replacing the serum with neonatal or fetal calf serum diminished any *in vivo* nutritional influences on lymphocyte blastogenic responses. Therefore, interpretation of *in vitro* studies and the results obtained may be related to culture preparation and may be critical in minimising the responses observed to different mitogens by different authors.

4.5. Conclusion

In conclusion, the results from this study identified that dietary Mo or Fe produced no significant effects on immune responses in growing lambs. Effects of dietary Mo and Fe on innate immunity were inconclusive due to methodology employed and lack of a CD_{11a}/CD_{18a} commercial monoclonal antibody. Dietary Fe reduced the antioxidant status of SOD and CP that may have caused an increased susceptibility to infection. Humoral immunity and lymphocyte blastogenic response were not affected by dietary treatment although liver Cu concentration was significantly reduced by dietary Mo and Fe, possibly altering haemopoesis.

Chapter 5

The effect of dietary molybdenum or iron on copper status, pituitary gland function, ovary morphology and trace element accumulation in ovary, pituitary, cerebellum and liver of growing lambs

5.1. Introduction

The results from chapters 3 and 4 suggest a systemic effect of Mo on Cu metabolism but provide no indication as to where this dietary Mo may accumulate once absorbed. Although mineral retention in organs of ruminant animals have previously been researched by other authors (Grace and Lee, 1990; Bires *et al.,* 1995; Haywood *et al.,* 1998), few studies have investigated the effects of dietary Mo or Fe on trace element accumulation and morphology in the brain, pituitary gland and ovary of ruminant animals. The known effects of dietary Mo on fertility in cattle have been reported previously (Munro, 1957; Phillippo *et al.,*1987b; Van Niekerk and Van Niekerk, 1989b) and therefore trace element accumulation may alter organ function and hormone or enzyme secretion from these specific sites. Work by Pott *et al.* (1999) found that Mo concentration increased linearly within the liver, kidney and muscle as dietary Mo intakes increased in sheep.

The formation of TM in the rumen has been well documented (Dick *et al.,* 1975: Suttle, 1991), but there is scarce information on what effects TM may have on systemic accumulation in tissues of ruminant animals. Haywood and Dincer (1997) found that sheep that had been treated intravenously with TTM following diets containing high levels of dietary Cu had raised Cu and Mo contents in the brain and elevated Mo in the pituitary, demonstrating that Mo and Cu had crossed the blood brain barrier. Work by Frank (1998) and Frank *et al.* (2000a) in the moose (*Alces alces*), found that alterations in Cu containing enzymes and clinical symptoms were similar to those often observed in cattle with a secondary Cu deficiency. In addition, high levels of Mo caused alterations in endocrine

function, with depressed thyroxine levels and hyperinsulinaemia in the moose (Frank *et al.*, 2000a).

It is not known if high levels of dietary Mo or Fe will cross the blood-brain barrier, similar to intravenous TTM administration, and whether or not dietary Mo or Fe may be retained in other tissues. Therefore, the aims of this study were to investigate the effects of the Cu antagonists, Mo or Fe on Cu metabolism and trace element accumulation in the pituitary, ovary, liver and cerebellum of growing lambs. In addition, their effects on pituitary endocrine function and pituitary and ovary morphology were investigated.

5.2. Materials and methods

5.2.1. Experimental Design and Animals

Fifty Charollais x (Suffolk x Mule) female lambs of approximately 12 weeks of age, with an initial mean live weight of 20.6 kg (s.e.d. 1.33) were used in this study. Lambs were randomly allocated to one of five dietary treatments (ten lambs per treatment group) (see section 5.2.2.) using a completely randomized design. The trial commenced on 26/2/01 for a period of twelve weeks. Lambs were housed in individual pens on elevated metal floors in a ventilated barn throughout the experimental period. Prior to the start of the trial, lambs were adapted from a pelleted lamb creep feed (S.C. Feeds, Stone, UK) containing 18% protein, 10% fibre, 4.5% oil and 8.5% ash onto the basal diet over a seven day adaptation period (see section 5.2.2.).

5.2.2. Diet Formulation

The raw materials used in this study were chosen because of their published low Cu and Mo contents (MAFF, 1992). Based on this information, the diets formulated were predicted to provide <5 mg kg^{-1} Cu DM. The basal diet was formulated to provide the metabolisable energy (ME) and metabolisable protein (MP) requirements of a 25 kg female lamb growing at 200g day^{-1} (AFRC, 1993) and had an ERDP of 94.57 kg^{-1} DM and FME of 9.92 MJ kg^{-1} DM.

Lambs were allocated by live weight to one of five dietary treatment groups which were:

Treatment one: Basal diet (Control)

Treatment two: Basal diet + 500 mg kg^{-1} DM iron and 2 g kg^{-1} DM sulphur (Fe)

Treatment three: Basal diet + 2 mg kg^{-1} DM molybdenum and 2 g kg^{-1} DM sulphur (2 Mo)

Treatment four: Basal diet + 5 mg kg^{-1} DM molybdenum and 2 g kg^{-1} DM sulphur (5 Mo)

Treatment five: Basal diet + 10 mg kg^{-1} DM molybdenum and 2 g kg^{-1} DM sulphur (10 Mo)

Feed samples were obtained from separate tote bags once weekly. All feed samples were analysed for dry matter, crude protein, neutral detergent fibre, ether extract, ash and neutral cellulase gamanase digestibility as described in sections 2.1.1. to 2.1.6. respectively. The raw material and chemical composition of the basal diet is presented in Table 5.1. Additional mineral inclusion per treatment diet is presented in Table 5.2.

Table 5.1: Diet formulation and analysed chemical composition of the basal diet (DM)

Ingredient (g kg^{-1})	Basal diet
Straw pellets (NaOH treated)	400
Whole barley	300
Rapeseed meal	200
Molasses	65
Mineral/vitamin Premix[1]	35
Total	1000
Dry matter (g kg^{-1} DM)	878.1
ME (MJ kg^{-1} DM)	10.56*
Crude protein (g kg^{-1} DM)	138.5
Neutral detergent fibre (g kg^{-1} DM)	435.5
Ether extract (g kg^{-1} DM)	15.7
Ash (g kg^{-1} DM)	90.86
NCGD (g kg^{-1} DM)	737.2

*Predicted value from equation given in AFRC (1993).
[1] Mineral premix – see Table 5.2.

Table 5.2. Additional mineral inclusion for the control, iron and molybdenum treatment diets (kg ton^{-1})

Ingredient	Control	Iron	2 Mo	5 Mo	10 Mo
			Treatment diet		
Ammonium molybdate (kg)[1]	-	-	0.0016	0.008	0.016
Iron sulphate (kg)[1]	-	2.15	-	-	-
Ammonium sulphate (kg)[1]	-	6.06	7.078	7.078	7.078
Urea (kg)[3]	3.2	0.46	-	-	-
Sand (kg)	6.8	1.33	2.918	2.914	2.906
Mineral premix[2]	25	25	25	25	25
Total (kg)	35	35	35	35	35

[1]BDH Laboratory Supplies, Poole, Dorset, UK
[2]Intensive lamb premix, 25kg ton^{-1} (Frank Wright Ltd, Ashbourne, Derbyshire, UK) containing 8 miu Vit A, 2.0 miu Vit D3, 30g Vit E, 50g Fe, 60g Mn, 2g Co, 60g Zn, 5g I, 0.4g Se, 2,500g Ammonium Chloride, 20.42 % Ca, 2 % P, 0.13 % S, 30 % salt
[3]Urea (Trouw Nutrition, Northwich, Cheshire, UK)

The mineral composition of the basal diet was determined by ICP-ES (see section 2.3). Molybdenum was supplemented as reagent grade ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$), iron was supplemented as reagent grade iron sulphate ($FeSO_4.7H_2O$), and additional sulphur was supplemented as reagent grade ammonium sulphate ($(NH_4)_2SO_4$) (all BDH Laboratory Supplies, Poole, Dorset, UK). The nitrogen content of the mineral mix was balanced with urea (Trouw Nutrition, Northwich, Cheshire, UK) and sand was used as an inert material to balance mass.

Table 5.3. Analysed mineral composition of the basal diet

Element	Content
Calcium (g kg^{-1})	8.30
Phosphorus (g kg^{-1})	3.80
Sulphur (g kg^{-1})	2.70
Magnesium (mg kg^{-1})	0.17
Copper (mg kg^{-1})	6.09
Molybdenum (mg kg^{-1})	0.94
Iron (mg kg^{-1})	194.22
Zinc (mg kg^{-1})	59.99
Cobalt (mg kg^{-1})	2.19
Selenium (mg kg^{-1})	0.09
Manganese (mg kg^{-1})	59.71

Fresh tap water was offered *ad libitum*. Copper content of the water was 35µg/l (Dee Valley Group Laboratory, Rhostyllen, Wrexham, UK).

5.2.3. Experimental Routine

All lambs were fed at 08:00 and 15:00 daily into individual feed boxes to prevent mineral contamination. All feed was weighed into individual feed buckets using metric scales, calibrated using metric standard weights (F.J. Thornton and Co. Ltd., Wolverhampton, UK). Food refusals were weighed back twice weekly (section 2.2.11.) on Monday and Thursdays to determine individual lamb food intakes. The quantity of feed offered was rationed and recalculated weekly according to the live weight of the lamb taken on the day of live weight determination (see section 5.2.3.1.) in order to achieve a growth rate of 200 g day^{-1}.

5.2.3.1. *Liveweight determination*

All lambs were weighed once weekly on Mondays at 11:00 using the Standard Operating Procedure as described in section 2.2.11. Daily Liveweight Gain (DLWG) was calculated using regression analysis.

5.2.3.2. *Blood collection and analysis*

Blood samples were collected once weekly on Tuesdays at 10:00 as described in section 2.2. Fresh whole blood was used to assess haematocrit (Hc) (section 2.2.1.) and the remaining blood prepared as described in section 2.2. and stored at -20°C for subsequent analysis. Frozen samples were thoroughly defrosted and whole blood was subsequently analysed for superoxide dismutase activity (SOD) (section 2.2.5.) and haemoglobin concentration (Hb) (section 2.2.3.), plasma analysed for plasma copper concentration (Pl-Cu) (section 2.2.6.), and serum analysed for ceruloplasmin activity (CP) (section 2.2.4). The intra assay coefficient of variation for respective standards were: SOD 3.38 %, Pl-Cu 5.59 %, Hb 2.14 % and CP 7.46 %.

5.2.4. *Necropsy*

All fifty lambs were sent to a commercial slaughterhouse after 13 weeks on trial. All lambs were electrically stunned and killed by exsanguination. Livers, ovaries, brains and pituitaries were removed and cleaned. The brains were removed following the method of McCurnin and Bassert (2002). The cerebellum, ovary and pituitary samples were immediately fixed in fresh 10% buffered formalin (HD Supplies, Aylesbury, Bucks, UK) and subsequently analysed for trace element concentration as described in section 2.2.9. Livers were stored in clean plastic bags at -20°C and subsequently analysed for trace element determination (see section 2.2.9). Only 25 of the 50 cerebellums were analysed for trace element determination.

5.2.5. Histology

Tissue samples were removed from the 10% neutral buffered formalin. Multiple pituitary and ovary tissue sections were processed in a Leitz Rotary Microtome 1512 (non-automated) tissue processor (Leica, UK) and cut 5 μm thick. Tissues were stained as described in section 5.2.5.1. Ovary sections were prepared from only the Control and 10 Mo dietary treatment groups. Pituitary sections from all dietary treatment groups were prepared.

5.2.5.1. Haematoxylin and Eosin (H+E) stain

Ovary and pituitary sections were placed in metal slide staining racks (R.A. Lamb, East Sussex, UK) deparaffinized for five minutes, hydrated and stained for five minutes in Mayer's haemalum (Merck, Darmstadt, Germany). All sections were then "blued" in running water for five minutes after which they were stained in 1% aqueous eosin with acetic acid for five minutes. After the final washing, all sections were dehydrated through graded alcohols (96% and 100% ethanol) and finally in xylene (BDH Laboratory Supplies, Poole, Dorset, UK) and mounted in DPX (a synthetic resin medium) (Merck, Darmstadt, Germany). Pituitary sections stained with Haematoxylin and Eosin (H+E) stain identified basophils with blue staining and acidophils with red staining.

5.2.5.2. Periodic Acid Schiff (PAS) / Orange G Method

Pituitary tissue sections were placed in metal slide staining racks (R.A. Lamb, East Sussex, UK) and treated with 1% periodic acid for ten minutes (Product 29460, Merck, Darmstadt, Germany). Sections were washed with distilled water and subsequently treated with Feulgen stain (Schiff) (Product 35120, Merck, Darmstadt, Germany) for twenty minutes. Tissue sections were then thoroughly washed in running tap water for five minutes, stained in Mayers Haemalum (Lillie, 1965) for two minutes and then "blued" in running tap water for five minutes. Sections were then stained in 2% orange G (Merck, Darmstadt,

125

Germany) in 5% phosphotungstic acid (Merck, Darmstadt, Germany) for twenty seconds and then placed in tap water until the section was pale yellow in colour, and then dehydrated through graded alcohols (96% and 100% ethanol) to xylene (BDH Laboratory Supplies, Poole, Dorset, UK) and mounted in DPX (a synthetic resin medium) (Merck, Darmstadt, Germany). Pituitary sections stained with PAS/orange G identified basophils with magenta staining, acidophils with yellow / pale orange staining, and chromophobes as pale blue / grey staining.

5.2.6. Adrenocorticotrophic Hormone (ACTH) immunohistochemistry on the pituitary gland

Pituitary gland tissue samples were fixed in 10% neutral buffered formalin as described in section 5.1.4. (prepared twice for positive and negative controls). This method employed the monoclonal mouse antibody (MAb) (DAKO Corporation, Carpinteria, California, USA) according to the method of A. Kipar (Department of Veterinary Pathology, University of Liverpool, UK, personal comm.). This method used immunohistochemistry (IHC) and the mouse monoclonal antibody to identify corticotrophs in the adenohypophysis that stained for anti-ACTH (adrenocorticotropin). Methods for the preparation of Phosphate Buffered Saline (PBS), Imidazole/HCL buffer 0.1M, DAB solution and Papanicolaou's stain are described in Appendix 3.

All tissue sections were placed in metal slide staining racks (R.A. Lamb, East Sussex, UK) and placed in xylene (BDH Laboratory Supplies, Poole, Dorset, UK) for deparaffination for five minutes, and then subsequently placed twice in 100% ethanol and once in 96% ethanol (BDH Laboratory Supplies, Poole, Dorset, UK) for rehydration. All samples were placed in methanol (containing freshly added 0.5% Hydrogen peroxide) (BDH Laboratory Supplies, Poole, Dorset, UK) and left at room temperature for thirty minutes. Tissue sections were then thoroughly washed in distilled water and placed into coverplates on Shandon Sequenza racks (Thermo Shandon, Runcorn, Cheshire, UK) before undergoing a further wash in distilled water for five minutes. For all positive tissue sample sections, 100

μl of a non-specific binding antiserum using 10% rat serum (Jackson ImmunoResearch Laboratories Inc., PA, USA) in PBS was carefully pipetted onto each section and left for ten minutes at room temperature. To all positive sample sections, 98 μl of ACTH Mab (1:20 in Tris buffered saline, see appendix 3) (DAKO Corporation, Carpinteria, California, USA) was pipetted onto each section and left to incubate overnight in the fridge at 4°C. For the negative controls, TBS was pipetted onto each section in place of the ACTH Mab. Following incubation, all slides were thoroughly washed in TBS (still in the Sequenza racks) for five minutes. To all positive and negative controls, 98μl of AffiniPure Rat Anti-Mouse IgG (1:100 in TBS) (Jackson ImmunoResearch Laboratories Inc., PA, USA) was added and incubated at room temperature for 30 minutes, and subsequently washed with TBS. To all sections (positive and negative controls), 98 μl of (1:500 in TBS) PAP Mouse (Mouse Peroxidase-Anti-Peroxidase) (Jackson ImmunoResearch Laboratories Inc., PA, USA) was carefully pipetted to each slide and incubated for a further 30 minutes at room temperature. All slides were thoroughly washed in TBS, and subsequently removed from the coverplates and placed in tissue section racks. All slides were then placed in a glass dish containing a magnetic stirrer and incubated for 10 minutes in 3,3'-diaminobencidintetrahydrochloride (DAB) (Fluka, Gillingham, Dorset, UK) containing 0.01% Hydrogen peroxide (30%) (BDH Laboratory Supplies, Poole, Dorset, UK) in 0.1M imidazole / HCl buffer 0.1M buffer (pH 7.1) at room temperature. Following incubation, all sections were thoroughly washed three times each for five minutes in TBS and once in tap water for five minutes. All slides were subsequently counterstained for 30 seconds with Pananicolaou's haematoxylin 1b stain (Merck, Darmstadt, Germany), followed by a final rinse in tap water. To complete the procedure, all slides were dehydrated sequentially in 96% ethanol, 100% ethanol and twice in xylene and cover slips placed carefully on each slide for subsequent analysis. All slides were initially subjectively assessed with the naked eye for ACTH reactivity and subsequently under the light microscope.

5.2.7. Statistical analysis

Statistical analysis was performed using Genstat version 5.0. All haematology and enzyme parameters and trace element concentrations were analysed by analysis of variance (ANOVA) and repeated measures (where appropriate) using a completely randomized design. Daily liveweight gain (DLWG) was determined by regression analysis. In all graphs, error bars refer to 2x s.e.d. Differences between treatments was calculated using a protected least significant difference (LSD) (Snedecor and Cochran, 1989).

5.3.   Results

5.3.1. Growth rate and performance characteristics

There was no effect of dietary treatment on lamb live weight at any time point (Fig 5.1.).

There was no significant effect of 2, 5 or 10 mg kg^{-1} Mo or 500 mg kg^{-1} Fe on total gain,

DLWG, daily dry matter intake (DMI), total DMI, or food conversion efficiency (FCE)

(Table 5.4.). The DLWG for all treatment groups was slightly less than the 200 g day^{-1} as

formulated by AFRC (1993) (Table 5.4.).

Table 5.4.   *Effect of molybdenum or iron supplementation on food intake and performance of growing lambs (kg)*

	Treatment		
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Sig
Initial wt	22.7	22.8	22.8	22.6	23.2	1.40	NS
Slaughter wt	40.9	39.9	40.6	41.1	40.3	2.17	NS
Total gain	18.2	17.1	17.7	18.5	17.1	1.18	NS
DLWG	0.19	0.18	0.19	0.19	0.18	0.012	NS
Daily DMI	1.47	1.44	1.47	1.48	1.48	0.061	NS
Total DMI	133.9	131.3	134.2	134.4	135.1	5.54	NS
FCE*	0.13	0.13	0.13	0.14	0.13	0.007	NS

*FCE calculated as Total gain (kg) divided by Total DMI (kg)
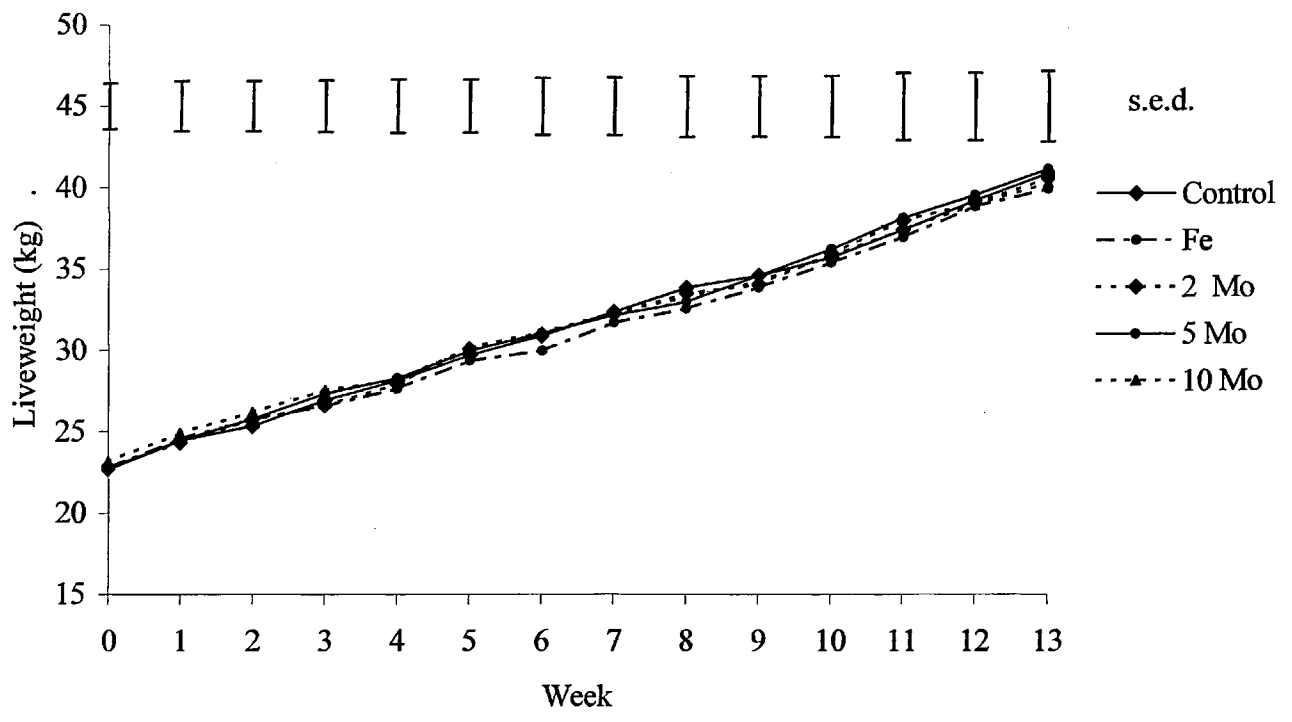
Figure 5.1. *Effect of molybdenum or iron supplementation on live weight of growing lambs*

5.3.2. Haematocrit

There was no significant effect of the 2, 5 or 10 mg kg^{-1} Mo or 500 mg kg^{-1} Fe dietary treatments on haematocrit (Hc) % in growing lambs during the experimental period (Table 5.5.). All Hc were considered to be within the normal reference ranges for lambs (Appendix 1). Repeated measures analysis indicated that there was no significant effect of dietary treatment or time x treatment interaction on Hc but there was an effect of time ($P<0.001$) on Hc in growing lambs (Table 5.5.).

Table 5.5. *Effect of molybdenum or iron supplementation on haematocrit (%) of growing lambs*

	Treatment						
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
Week							
0	36.4	36.7	37.6	36.3	38.0	1.33	NS
2	35.2	35.1	35.4	35.0	35.5	1.22	NS
4	34.1	35.5	34.6	34.4	35.1	1.04	NS
6	32.4	35.3	33.9	32.9	34.4	1.08	NS
8	32.8	35.0	33.7	32.6	33.3	1.33	NS
10	31.8	34.2	32.7	30.8	33.2	1.15	NS
12	32.2	32.8	34.0	31.2	32.1	1.22	NS

Repeated Measures Analysis		
	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

5.3.3. Haemoglobin Concentration

There was no effect of the 2, 5 or 10 mg kg^{-1} Mo or 500 mg kg^{-1} Fe treatments on Hb concentration in growing lambs during the experimental period (Table 5.6.). All Hb concentrations were considered to be within the normal reference ranges for lambs (Appendix 1). Repeated measures analysis indicated that there was no effect of dietary treatment and no time x treatment interaction on Hb concentration but there was an effect of time on Hb concentration in growing lambs.

Table 5.6. *Effect of molybdenum or iron supplementation on haemoglobin concentration (g/dl) in growing lambs*

	Treatment						
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
Week							
0	11.9	11.1	11.2	11.2	12.3	0.71	NS
2	13.0	12.9	12.0	13.1	13.3	0.62	NS
4	12.1	12.4	12.0	12.3	12.3	0.41	NS
6	13.5	14.2	13.1	13.8	13.8	0.91	NS
8	11.4	11.7	11.7	10.8	11.3	0.54	NS
10	12.7	12.5	11.7	12.0	12.1	0.78	NS
12	13.7	13.3	13.3	12.0	13.7	1.09	NS

Repeated Measures Analysis: Treatment effect   P = NS

Time effect   P = <0.001

Time X Treatment effect   P = NS

5.3.4. Plasma Copper Concentration

Lambs that received the 10 mg kg^{-1} Mo dietary treatment had higher Pl-Cu concentrations compared to all other dietary treatment groups at week 6 (P<0.05), week 8 (P<0.05) and week 12 (P<0.001) (Fig. 5.2.). There were no differences in Pl-Cu concentration between the lambs that received the control, 500 mg kg^{-1} Fe, 2 mg kg^{-1} Mo or 5 mg kg^{-1} Mo dietary treatments at any time point. There was a sharp increase in the mean Pl-Cu concentrations between week 10 and 12 in lambs that received the 10 mg kg^{-1} Mo dietary treatment whilst Pl-Cu concentrations of all other treatment groups remained similar to Pl-Cu concentrations from the previous week. Repeated measures analysis indicated that there was an effect of treatment (P<0.01), a time x treatment interaction (P<0.001) and an effect (P<0.001) of time on Pl-Cu concentration. These differences were due to the increased Pl-Cu concentrations in the 10 Mo dietary treatment group compared to all other dietary treatment groups.
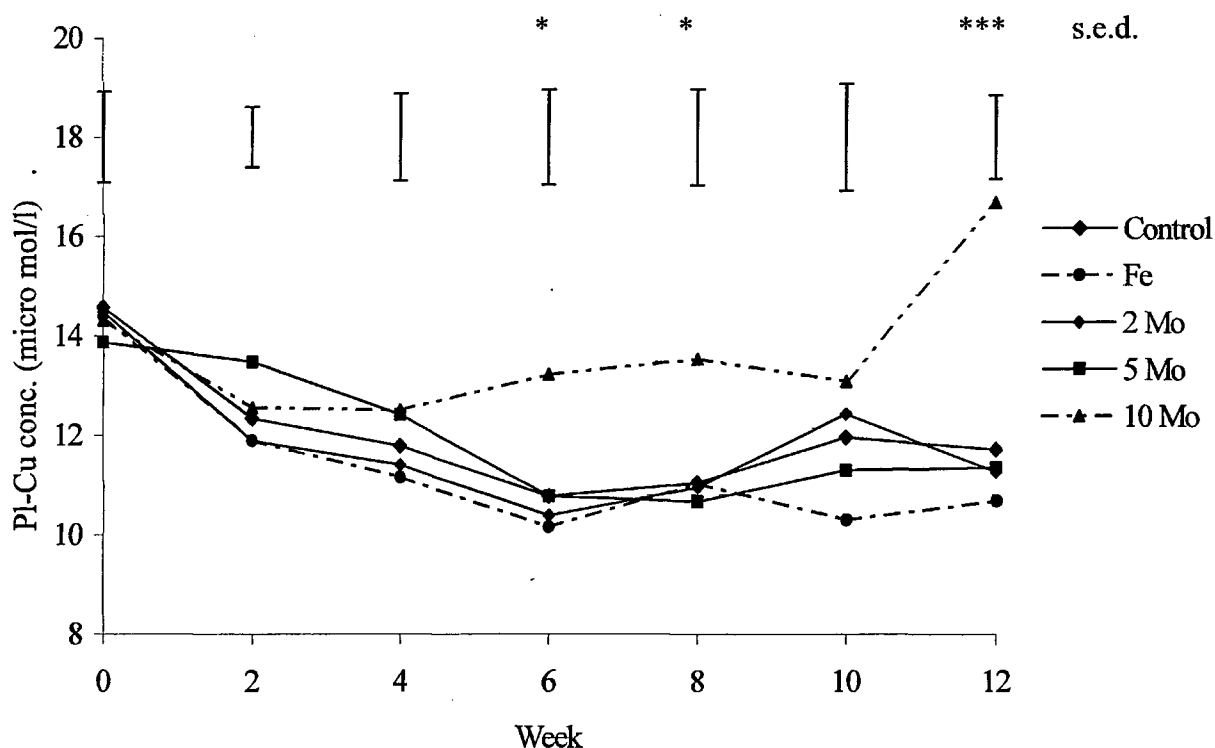
Figure 5.2.   *Effect of molybdenum or iron supplementation on plasma copper concentration of growing lambs*

Repeated Measures Analysis	Treatment effect	P = <0.01
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.001

5.3.5. Ceruloplasmin Activity

There was no significant effect of dietary treatment on ceruloplasmin (CP) activity at any time point from weeks 2 to 10 inclusive (Fig 5.3.). At week 12, the lambs that received the 5 mg kg^{-1} Mo dietary treatment diet had a lower (P<0.05) CP activity compared to the lambs that received the control, 500 mg kg^{-1} Fe, 2 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments. Repeated measures analysis indicated that there was no effect of dietary treatment and no time x treatment interaction but there was an effect of time (P<0.001) on mean CP activity. This was due to the decrease in CP activity from week 0 to week 12 in all treatment groups.
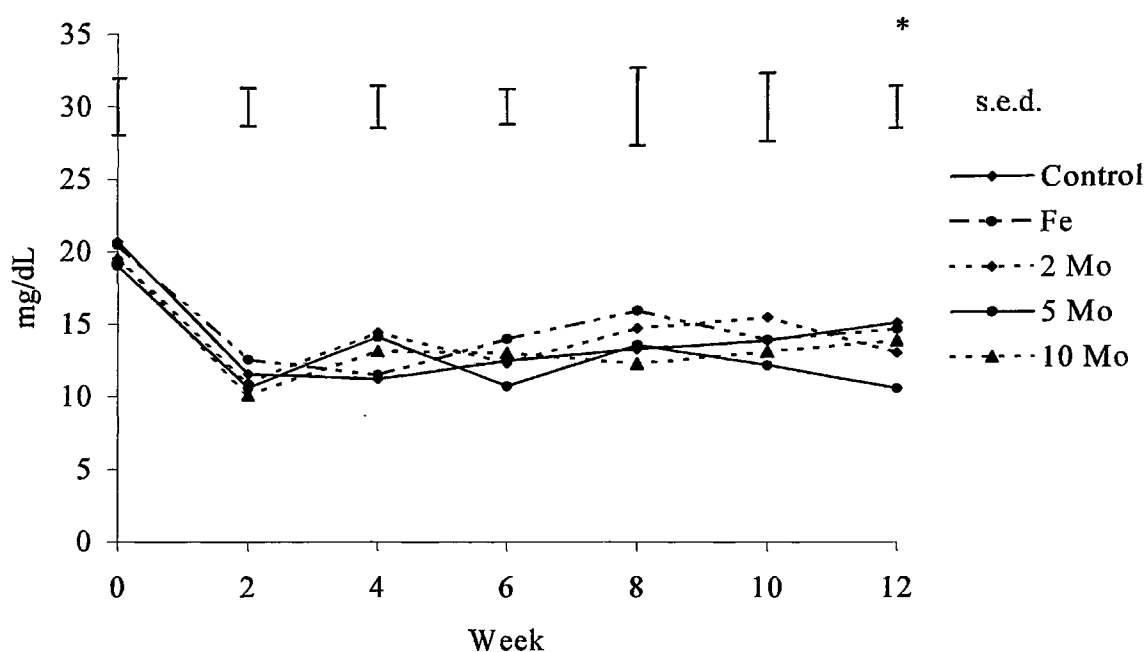
Figure 5.3.   *Effect of molybdenum or iron supplementation on ceruloplasmin activity of growing lambs*

Repeated Measures Analysis	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

5.3.6. *Ceruloplasmin to Plasma Copper ratio (CP:Pl-Cu)*

At week 2, lambs that received the 5 mg kg^{-1} Mo dietary treatment had a lower (P<0.01) CP:Pl-Cu ratio compared with the lambs that received the control or 500 mg kg^{-1} Fe dietary treatments (Table 5.7.). In addition, at week 2, lambs that received the 10 mg kg^{-1} Mo dietary treatment had a lower (P<0.01) CP:Pl-Cu ratio compared with the lambs that received the 500 mg kg^{-1} Fe dietary treatment. At week 6, the lambs that received the 500 mg kg^{-1} Fe dietary treatment had a higher (P<0.001) CP:Pl-Cu ratio compared to all other dietary treatment groups. At week 6, lambs that received the 5 or 10 mg kg^{-1} Mo dietary treatments had lower CP:Pl-Cu ratios compared to lambs that received the control, 500 mg kg^{-1} Fe or 2 mg kg^{-1} Mo dietary treatments. At week 8, lambs that received the 500 mg kg^{-1} Fe dietary treatment had a higher (P<0.01) CP:Pl-Cu ratio compared to lambs that received the 2, 5 or 10 mg kg^{-1} Mo dietary treatments. Repeated measures analysis indicated that there was an effect (P<0.001) of dietary treatment, an effect of time (P<0.001) and a time x treatment interaction (P<0.05) on CP:Pl-Cu ratio. The CP:Pl-Cu ratio decreased in all dietary treatment groups over time, particularly in the lambs that received the 5 mg kg^{-1} Mo dietary treatment.

Table 5.7.   *Effect of molybdenum or iron supplementation on ceruloplasmin to plasma copper ratio (CP:Pl-Cu) of growing lambs*

Week	Treatment						
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
0	1.34	1.47	1.29	1.27	1.36	0.086	NS
2	0.94^{ac}	1.04^{a}	0.92^{b}	0.78^{bc}	0.80^{abc}	0.075	**
4	0.97	1.05	1.29	1.15	1.10	0.147	NS
6	1.18^{b}	1.41^{c}	1.19^{a}	0.99^{a}	0.98^{ab}	0.057	***
8	1.21^{ab}	1.48^{b}	1.16^{a}	1.04^{a}	0.91^{ab}	0.149	**
10	1.16	1.23	1.22	1.02	1.01	0.081	NS
12	1.17	1.37	1.11	0.86	1.16	0.165	NS

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, *** <0.001

135

Repeated Measures Analysis	Treatment effect	P = <0.001
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.05

5.3.7. Superoxide Dismutase Activity

There was no effect of treatment on superoxide dismutase (SOD) activity at any timepoint throughout the trial (Fig 5.4.). Although there were fluctuations in SOD activity over the trial period, these changes followed a similar pattern for all treatments. Repeated measures analysis indicated that there was no effect of treatment and no time x treatment interaction, but there was an effect of time (P<0.001) on SOD activity. This could be seen by the decrease in SOD activity in all treatment groups over time, except for the sharp increase in SOD activity between weeks 6 and 8.
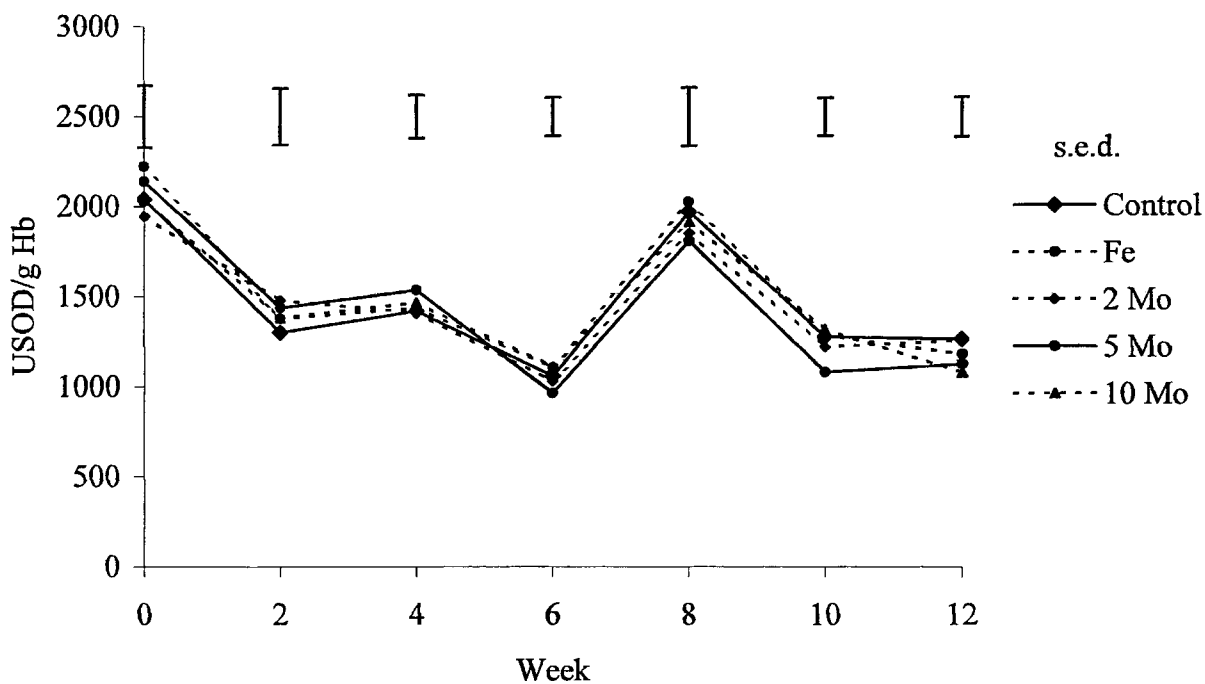
Figure 5.4.   *Effect of molybdenum or iron supplementation on superoxide dismutase activity of growing lambs*

Repeated Measures Analysis	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

5.3.8. *Trace element accumulation in the liver*

Liver Cu concentrations were higher (P<0.001) in lambs that received the control dietary treatment compared to all other dietary treatment groups (Table 5.8.). Lambs that received the 5 mg kg^{-1} Mo dietary treatment had lower (P<0.001) Cu concentrations in the liver compared with lambs that had received the control, 500 mg kg^{-1} Fe, 2 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments. Lambs that received the 10 mg kg^{-1} Mo dietary treatment had higher (P<0.001) liver Mo concentrations compared to all other dietary treatment groups. Lambs that received the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.001) liver Mo concentrations compared to lambs that received the control, 500 mg kg^{-1} Fe and 2 mg kg^{-1} Mo dietary treatments. Liver iron concentration was higher (P<0.001) in lambs that received the 500 mg kg^{-1} Fe dietary treatment compared to all other dietary treatments. There were no differences in liver iron concentration between the control, 2 mg kg^{-1} Mo, 5 mg kg^{-1} Mo or 10 mg kg^{-1} Mo dietary treatments. There was no effect of dietary treatment on zinc concentration in the liver.

Table 5.8.   *Effect of molybdenum or iron supplementation on trace element accumulation in the liver of growing lambs (μg/g DM $\pm$ s.e.d.)*

	Treatment						
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
Copper	215.1^c	82.5^b	77.7^{ab}	29.4^a	82.1^b	26.64	***
Molybdenum	3.76^b	3.51^{ab}	4.21^{bc}	4.94^c	6.77^d	0.32	***
Zinc	106.1	102.4	109.2	114.7	115.1	6.90	NS
Iron	203.0^a	520.1^b	201.2^a	206.1^a	189.0^a	35.5	***

^{a,b,c} Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, ** <0.01, *** <0.001

5.3.9. Trace element accumulation in the ovary

Lambs that received the 10 mg kg^{-1} Mo dietary treatment had a higher (P<0.05) ovary Cu concentrations compared to lambs that had received the control, 500 mg kg^{-1} Fe, 2 mg kg^{-1} Mo or 5 mg kg^{-1} Mo dietary treatments (Table 5.9.). There were no differences in ovary Cu concentration between the lambs that received the control, 500 mg kg^{-1} Fe, 2 mg kg^{-1} Mo or 5 mg kg^{-1} Mo dietary treatments. Lambs that received the 10 mg kg^{-1} Mo dietary treatment had a higher (P<0.001) ovary Mo concentration compared to lambs that had received the control, 500 mg kg^{-1} Fe, 2 mg kg^{-1} Mo or 5 mg kg^{-1} Mo dietary treatments. Lambs that received the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.001) ovary Mo concentration compared to lambs that received the control, 500 mg kg^{-1} Fe or 2 mg kg^{-1} Mo dietary treatments. There were no effects of dietary treatment on ovary Zn concentration. Lambs that received the 500 mg kg^{-1} Fe dietary treatment had higher (P<0.05) ovary Fe concentrations compared to the 2 mg kg^{-1} Mo and 10 mg kg^{-1} Mo dietary treatments.

Table 5.9.   Effect of molybdenum or iron supplementation on trace element accumulation in the ovary of growing lambs (µg/g DM ± s.e.d.)

	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
	Treatment						
Copper	7.02[a]	6.30[a]	5.55[a]	6.35[a]	8.05[b]	0.730	*
Molybdenum	0.24[a]	0.33[a]	0.30[a]	0.48[b]	0.85[c]	0.067	***
Zinc	17.46	19.41	19.98	20.82	19.49	2.208	NS
Iron	31.7[ab]	40.4[b]	26.6[a]	32.2[ab]	27.0[a]	4.42	*

[a,b,c] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, ** <0.01, *** <0.001

5.3.10. Trace element accumulation in the cerebellum

There were no significant effects of dietary treatment on copper, molybdenum, zinc or iron accumulation in the cerebellum of growing lambs (Table 5.10.).

Table 5.10.   *Effect of molybdenum or iron supplementation on trace element accumulation in the cerebellum of growing lambs (µg/g DM ± s.e.d.)*

	Treatment						
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
Copper	15.21	14.21	11.86	12.61	13.40	2.389	NS
Molybdenum	0.15	0.07	0.06	0.17	0.14	0.037	NS
Zinc	62.20	61.12	57.83	58.01	53.76	7.490	NS
Iron	97.26	78.85	72.38	101.18	124.05	37.902	NS

n = 5 lambs per treatment group
[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, ***<0.001

5.3.11. Trace element accumulation in the pituitary gland

There were no significant effects of dietary treatment on copper, molybdenum, zinc or iron accumulation in the pituitary gland of growing lambs (Table 5.11.).

Table 5.11.   *Effect of molybdenum or iron supplementation on trace element accumulation in the pituitary of growing lambs (µg/g DM ± s.e.d.)*

	Treatment						
	Control	Fe	2 Mo	5 Mo	10 Mo	s.e.d.	Significance
Copper	11.43	13.05	10.07	12.36	12.81	2.214	NS
Molybdenum	0.43	0.50	0.49	0.48	0.59	0.089	NS
Zinc	43.3	51.0	36.3	40.2	63.5	11.86	NS
Iron	150.0	152.1	135.1	153.0	166.0	33.30	NS

5.4. Histopathology – Ovary

Ovary sections were examined for identification of primordial follicles, primary and secondary follicles and atretic follicles and graded according to number of follicles counted (Table 5.12.). Example slides of follicle types are presented in Figs. 5.5 to 5.8. Through general observation, the number of primordial follicles present in the ovary of the control animals was generally higher than the lambs that received the 10 mg kg^{-1} Mo dietary treatment. The proportion of primary follicles was the same for both treatment groups. Atretic follicles in the ovary were more abundant in the lambs that received the 10 mg kg^{-1} Mo dietary treatment compared to the lambs that received the control treatment diet.

Table 5.12. *Ovary histopathology of lambs fed a control or 10 Mo dietary treatment*

Lamb no.	Treatment	Primordial follicle	Primary follicle	Secondary follicle	Atretic follicle
14	Control	+	++	++	+
52	Control	+	+	+++	+
105	Control	+++	++	+	+
119	Control	++	+	++	+
121	Control	++	+	+++	-
125	Control	+++	+++	+	+++
156	Control	++	+	+	+
Average[1]	*Control*	2.0	1.5	1.8	1.1
7	10 Mo	+	+	+	+
15	10 Mo	+++	+	+	++
53	10 Mo	+++	++	++	+
63	10 Mo	+	++	++	++
70	10 Mo	++	+	+	+
73	10 Mo	++	+	+	+
104	10 Mo	+	+	++	++
123	10 Mo	+	+++	++	++
158	10 Mo	+	+	++	+
162	10 Mo	+	+	+	+
Average[1]	10 Mo	1.6	1.4	1.5	1.4

Follicle number: (none - ; <5 + ; 6-10 ++ ; 11 plus +++)
[1] Average: No. of follicles / no. of samples
Number of subjects used: Control (7) and 10 Mo (10)
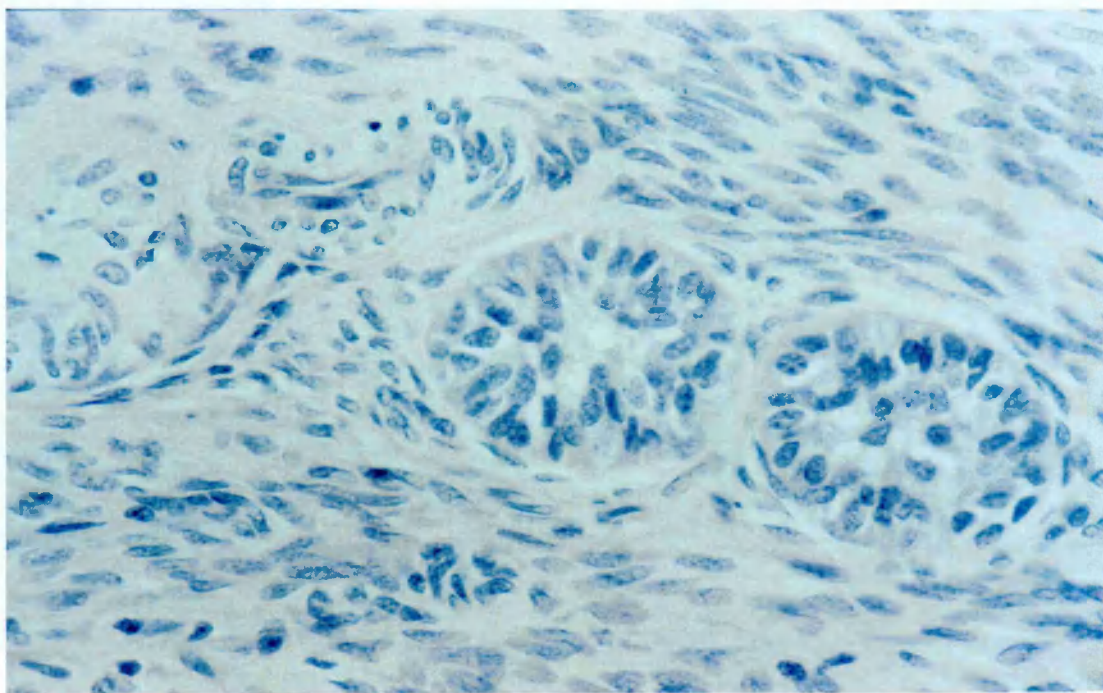
Fig. 5.5.   *Light microscope ovary section: Ovarian primary follicle (lamb tag no. 125, control treatment) (H+E) x 40*
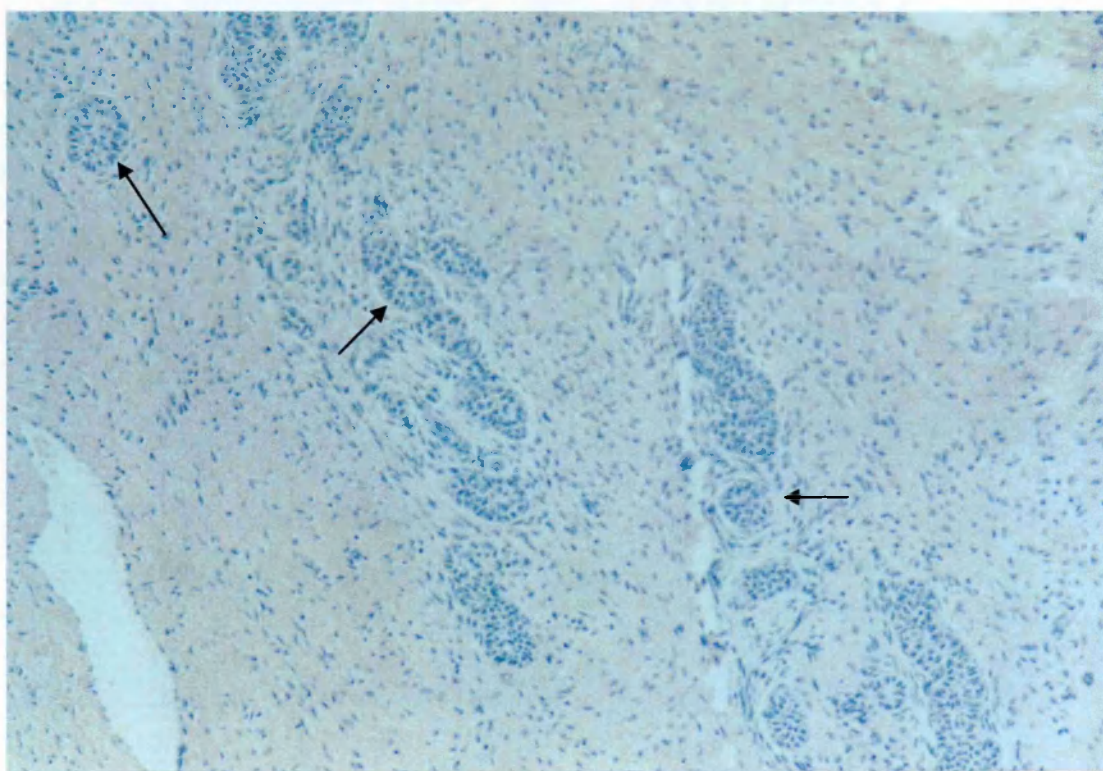
Fig. 5.6.   *Light microscope ovary section: Ovarian primary follicle (lamb tag no. 53, 10 Mo treatment) (H+E) x 80*
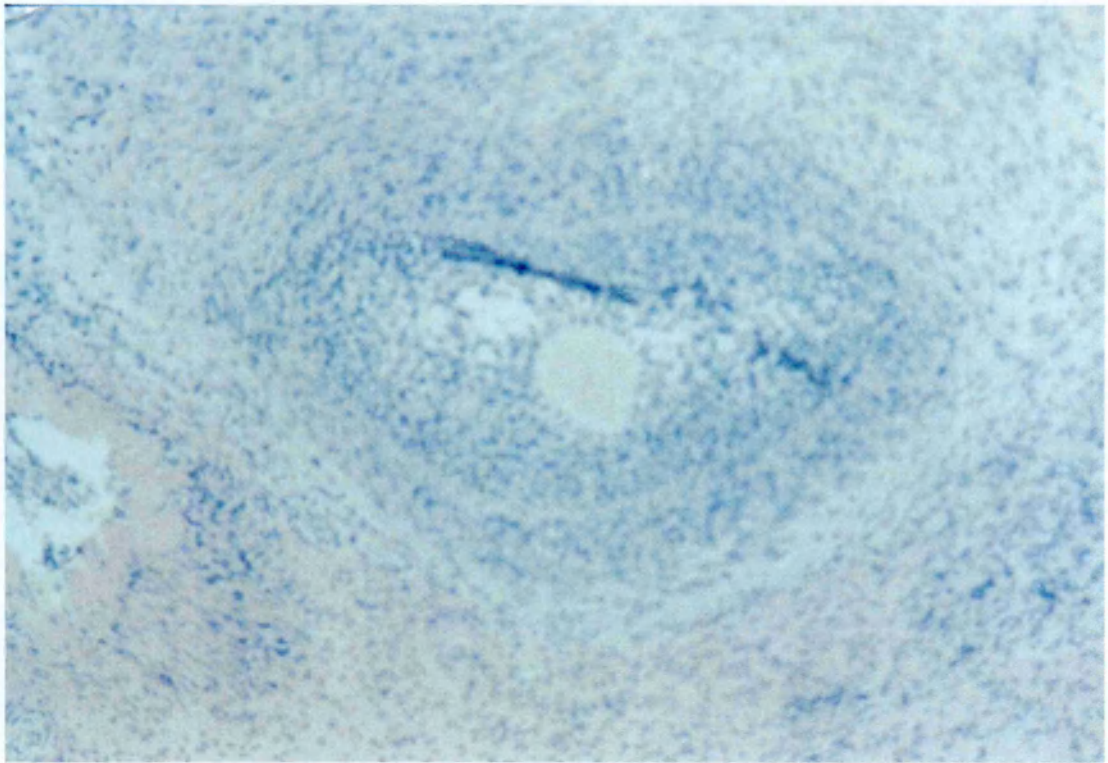
Fig. 5.7.   Light microscope ovary section: Ovarian atretic follicle
              (lamb tag no. 156, control treatment) (H+E) x 40
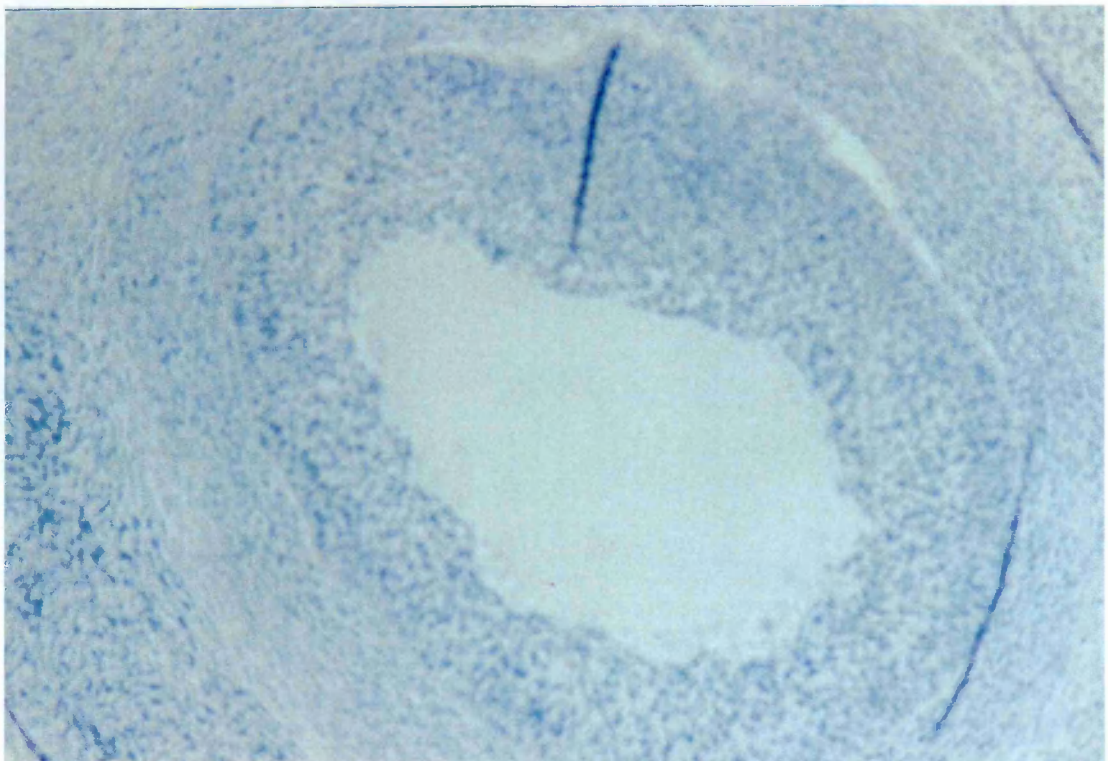
Fig. 5.8.   Light microscope ovary section: Ovarian atretic follicle
              (lamb tag no. 53, 10 Mo treatment) (H+E) x 40

5.5. Histopathology – pituitary gland

Sections stained with H+E stain identified basophils with blue staining (haematoxylin) and acidophils with red staining (eosin). Sections stained with PAS-Orange G identified basophils with magenta staining, acidophils with yellow/pale orange staining and chromophobes as pale blue/grey staining.

There was no obvious effect of dietary treatment on basophil and acidophil populations when stained with H+E (Fig. 5.9a to 5.13b inclusive). In the example slides (Fig. 5.9a to 5.13a inclusive) the proportion of basophils to acidophils was greater in the lambs that received the control dietary treatment, a greater number of acidophils to basophils in the lambs that received the 500 mg kg^{-1} Fe dietary treatment and proportionate numbers of basophil to acidophil populations in the lambs that received the 2, 5 and 10 mg kg^{-1} Mo dietary treatments. There was a large variation between slides from lambs that received the same dietary treatment so no conclusive effect of dietary treatment on pituitary gland histopathology could be made. This large variation between sections was also apparent when PAS-Orange G was used.
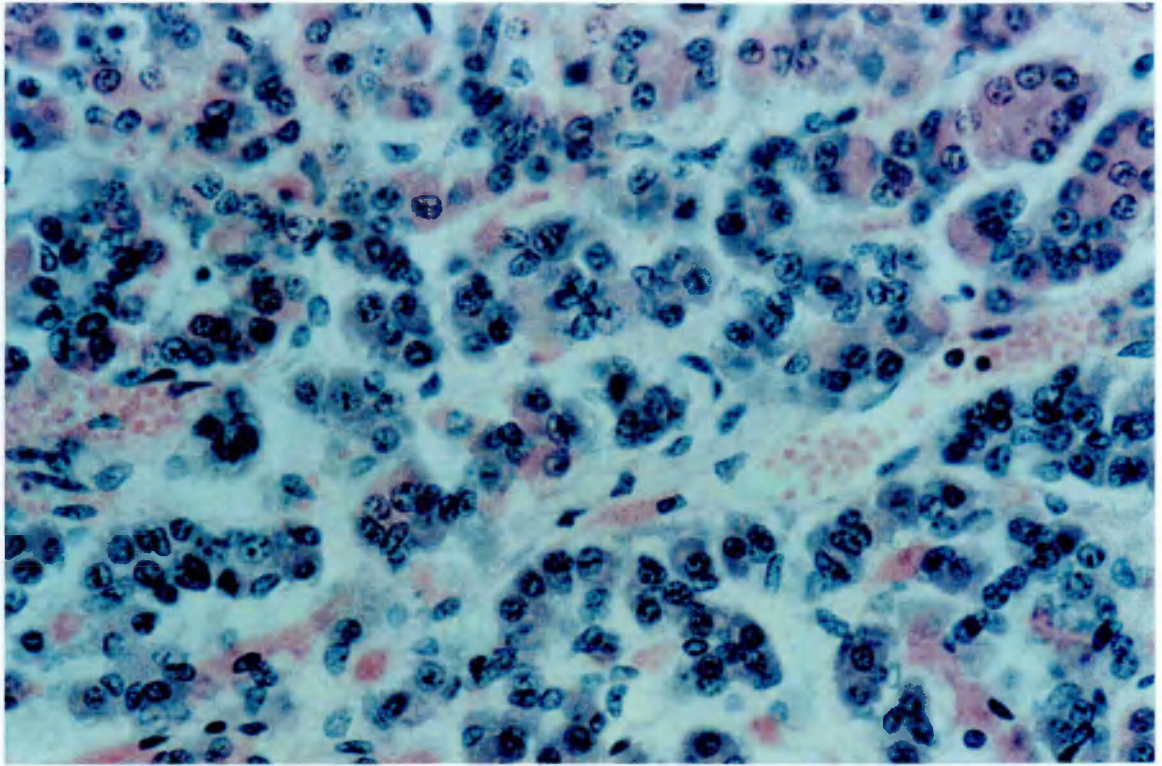
Fig 5.9.(a) Light Microscope pituitary section from lamb 64 (control treatment): Dark blue basophils (glycoprotein contaning cells) in abundance compared to red acidophils, H+E, x 40
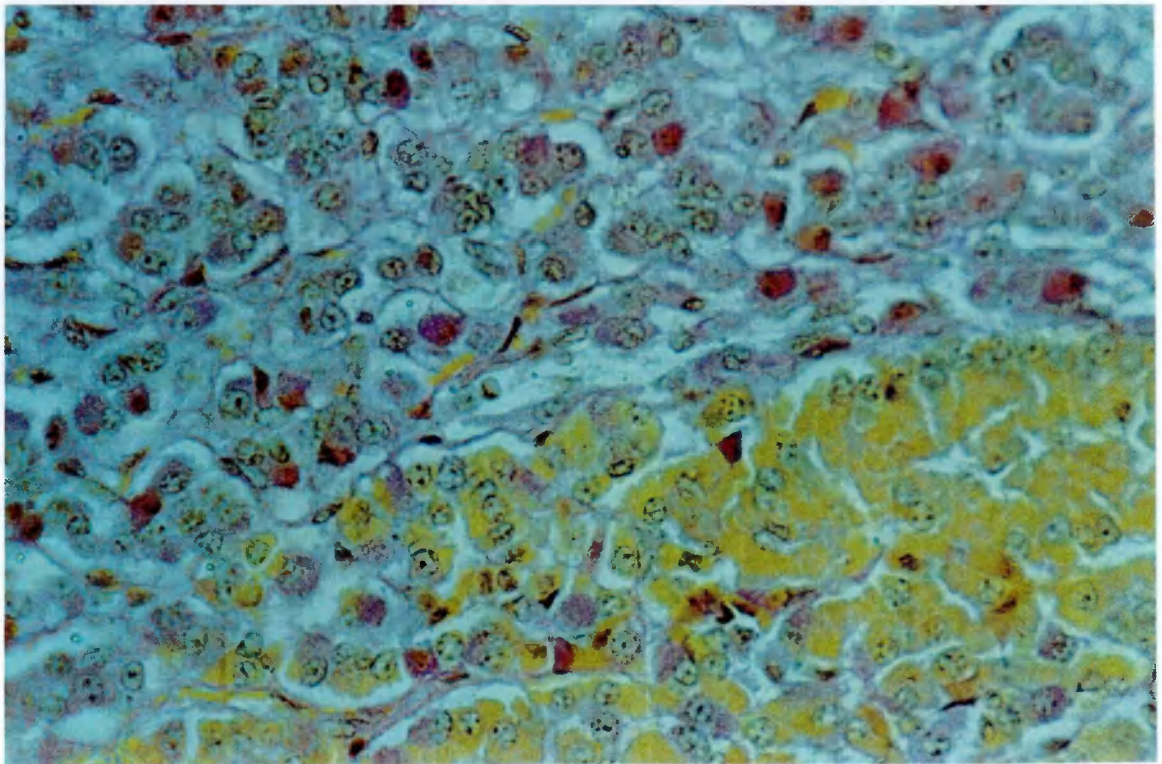
Fig 5.9.(b) Light Microscope pituitary section from lamb 64 (control treatment): Large population of somatotrophs (or acidophils) (yellow-orange) to glycoprotein-producing (basophil) cells (ACTH,LH,FSH,TSH) (magenta) x 40

144
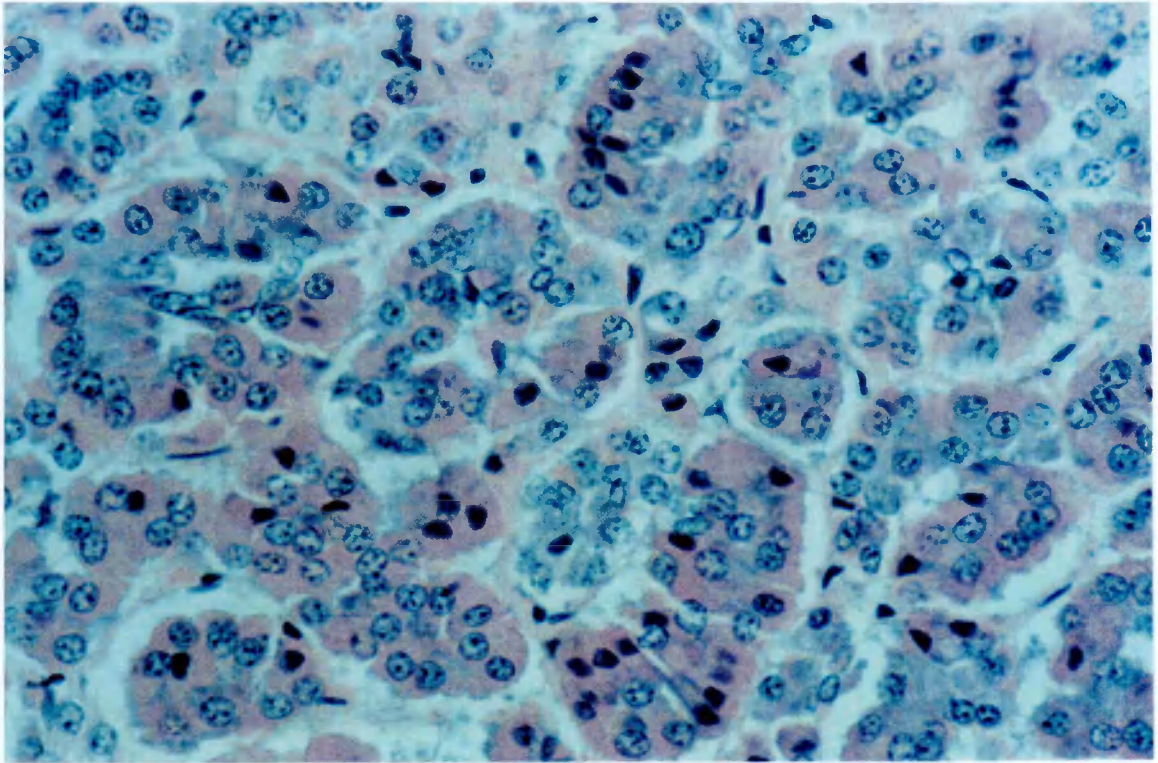
Fig 5.10.(a) Light Microscope pituitary section from lamb 127 (Fe treatment): Red acidophils in abundance compared to dark blue basophils (glycoprotein producing cells), H+E, x 40
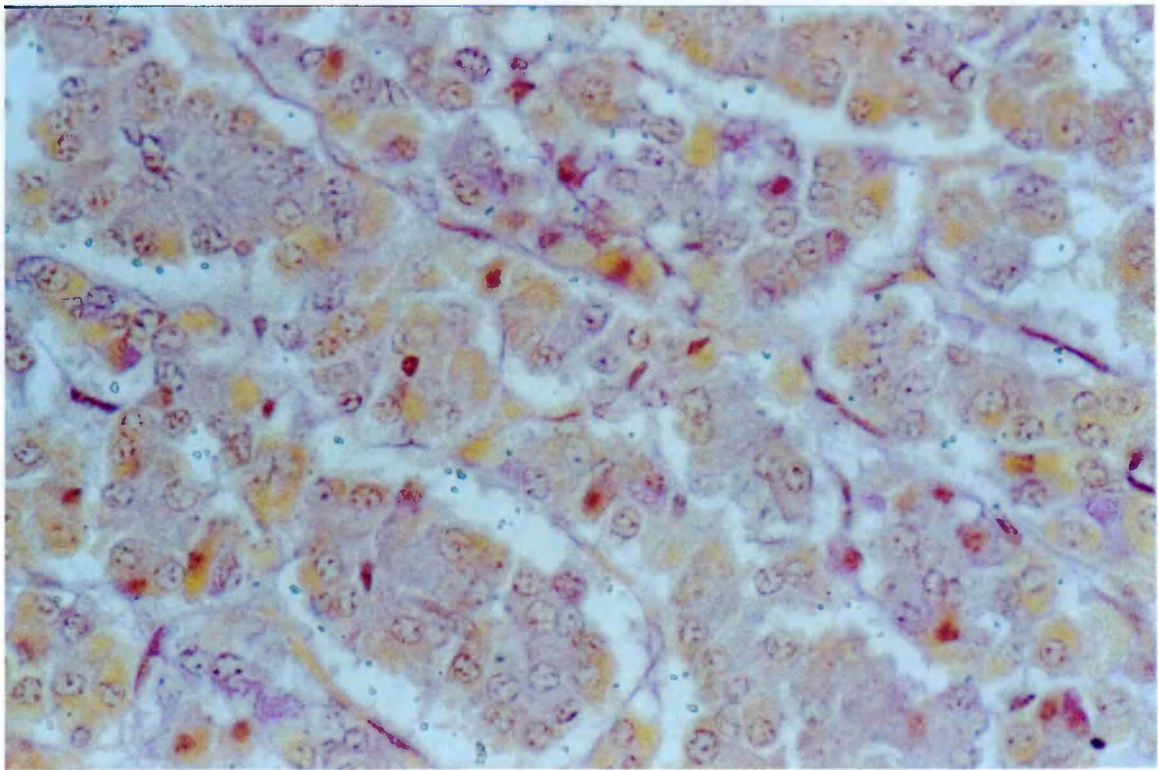
Fig 5.10.(b) Light Microscope pituitary section from lamb 127 (Fe treatment): Proportionate number of basophils (magenta) to acidophils (yellow-orange) x 40

145
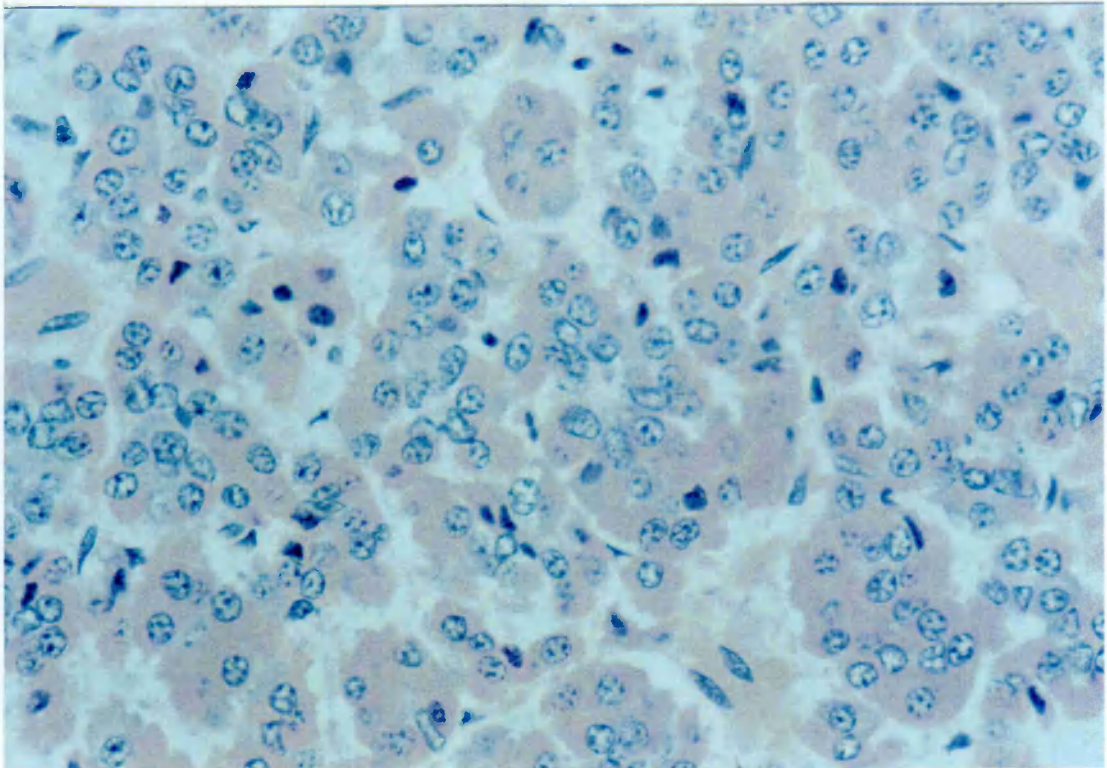
Fig 5.11.(a) Light Microscope pituitary section from lamb 166 (2 Mo treatment): Dark blue basophils (glycoprotein contaning cells) in proportion to red acidophils, H+E, x 40
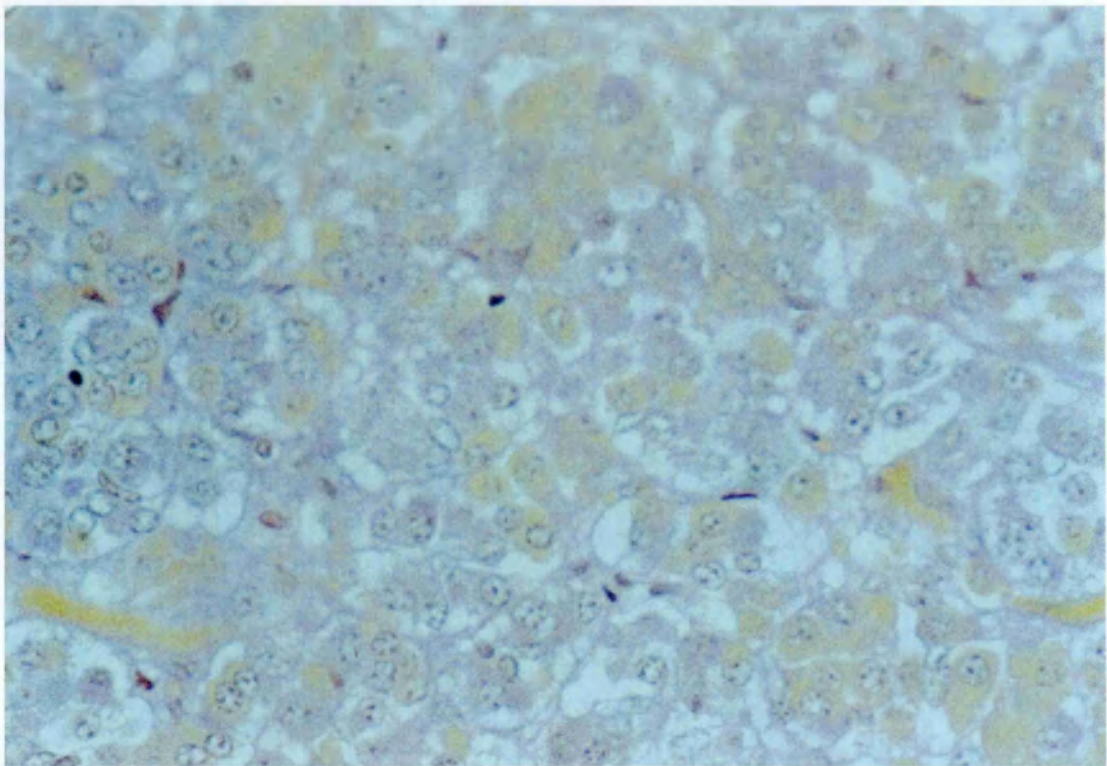
Fig 5.11.(b) Light Microscope pituitary section from lamb 166 (2 Mo treatment): Large proportion of glycoprotein-producing basophils (magenta) to acidophils (yellow-orange) x 40
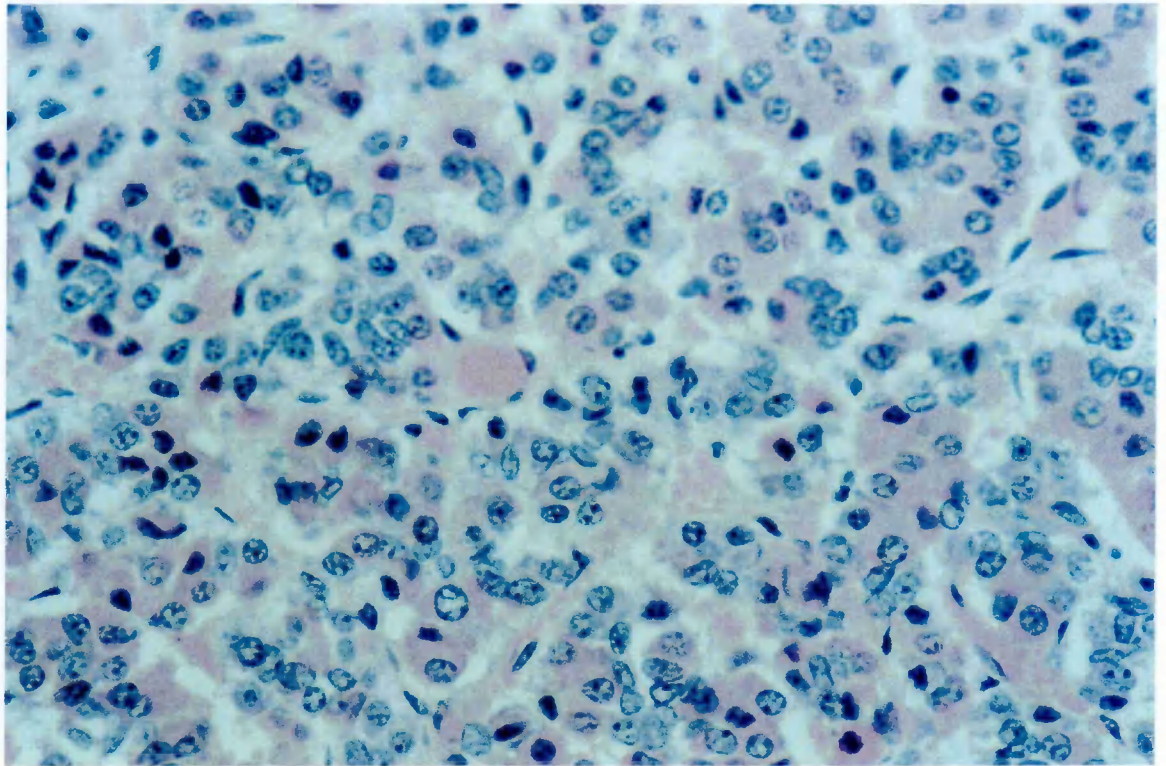
Fig 5.12.(a) Light Microscope pituitary section from lamb 26 (5 Mo treatment): Proportionate number of dark blue basophils (glycoprotein contaning cells) to red acidophils, H+E, x 40
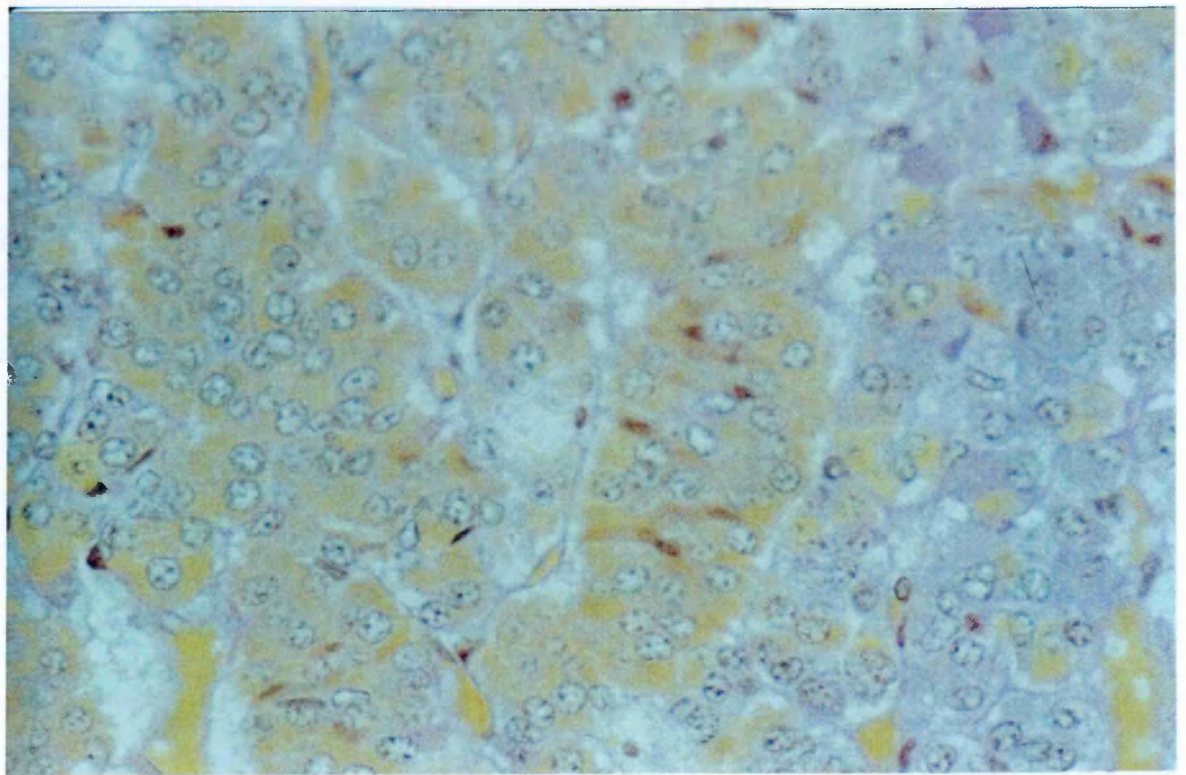
Fig 5.12.(b) Light Microscope pituitary section from lamb 26 (5 Mo treatment): Disproportionate number of acidophils (yellow-orange) to basophils (magenta) and large number of chromophobes (grey/pale blue), H+E, x 40

147
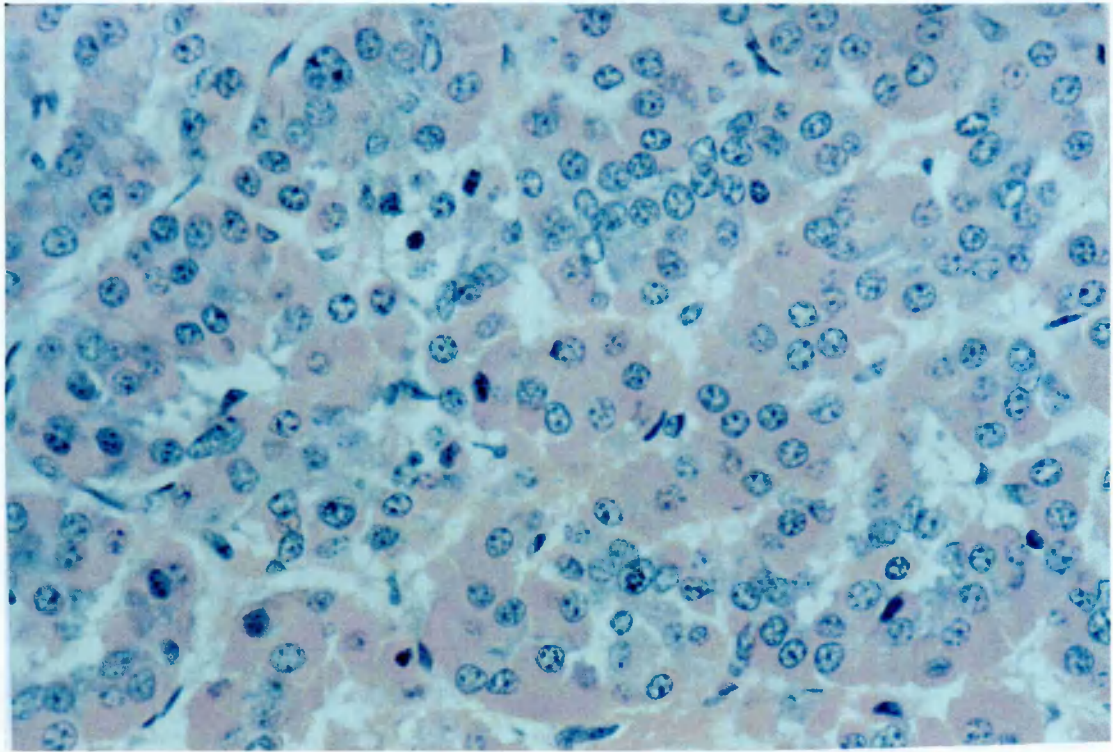
*Fig 5.13.(a) Light Microscope pituitary section from lamb 162 (10 Mo treatment)
: Proportionate number of dark blue basophils (glycoprotein contaning
cells) compared to red acidophils, H+E, x 40*
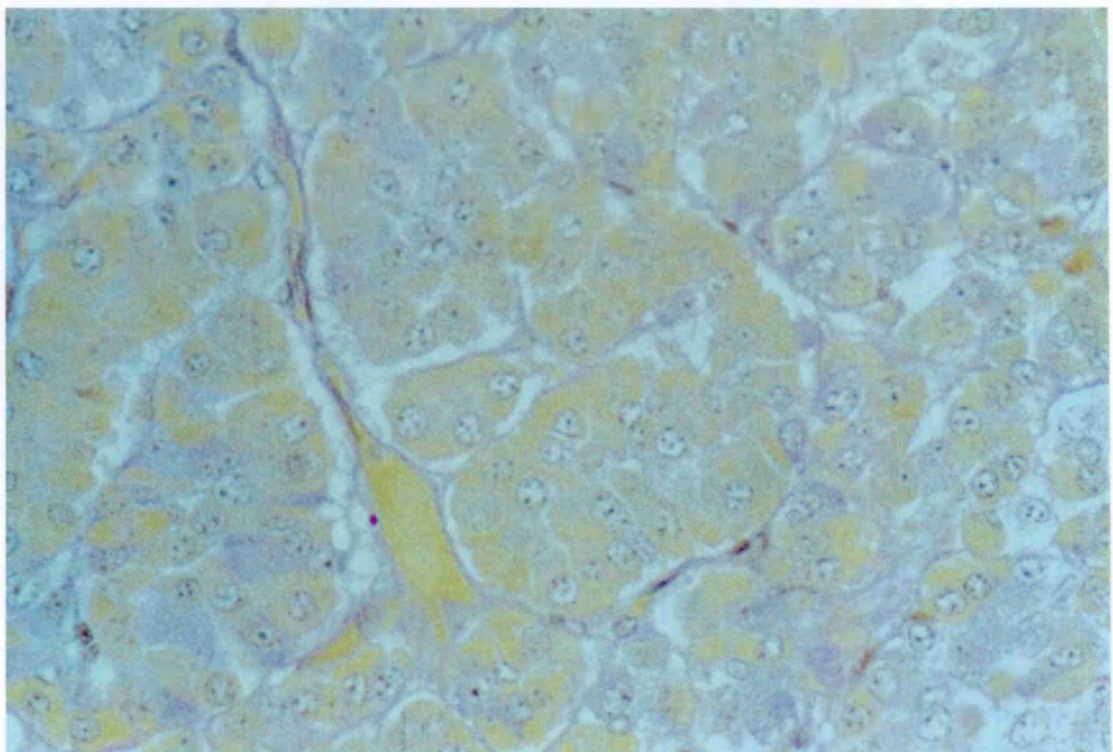
*Fig 5.13.(b) Light Microscope pituitary section from lamb 162 (10 Mo treatment):
Proportionate number of acidophils (yellow-orange) to basophils (magenta) and areas
of chromophobes (grey/pale blue), H+E, x 40*

148

5.6. *Immunohistochemistry – Adrenocorticotrophic Hormone (ACTH)*

ACTH immunostaining of pituitary samples was significantly darker and more intense in the lambs that received the Mo dietary treatment compared to the lambs that received the control diet or 500 mg kg^{-1} Fe treatment (Fig. 5.14 to 5.18 respectively). ACTH immunostaining of the (+) control samples from the control lambs was pale (Fig. 5.14a,b). Figs. 5.15(b) to 5.18(b) show a gradual increase in the intensity of ACTH immunostaining as dietary Mo is increased in the diet. Fig. 5.18 (b) from the 10 mg kg^{-1} Mo lambs illustrates ACTH accumulating in significant proportions in small, discrete areas of the pituitary gland when compared to Fig. 5.18 (a) from the respective control lambs.

149
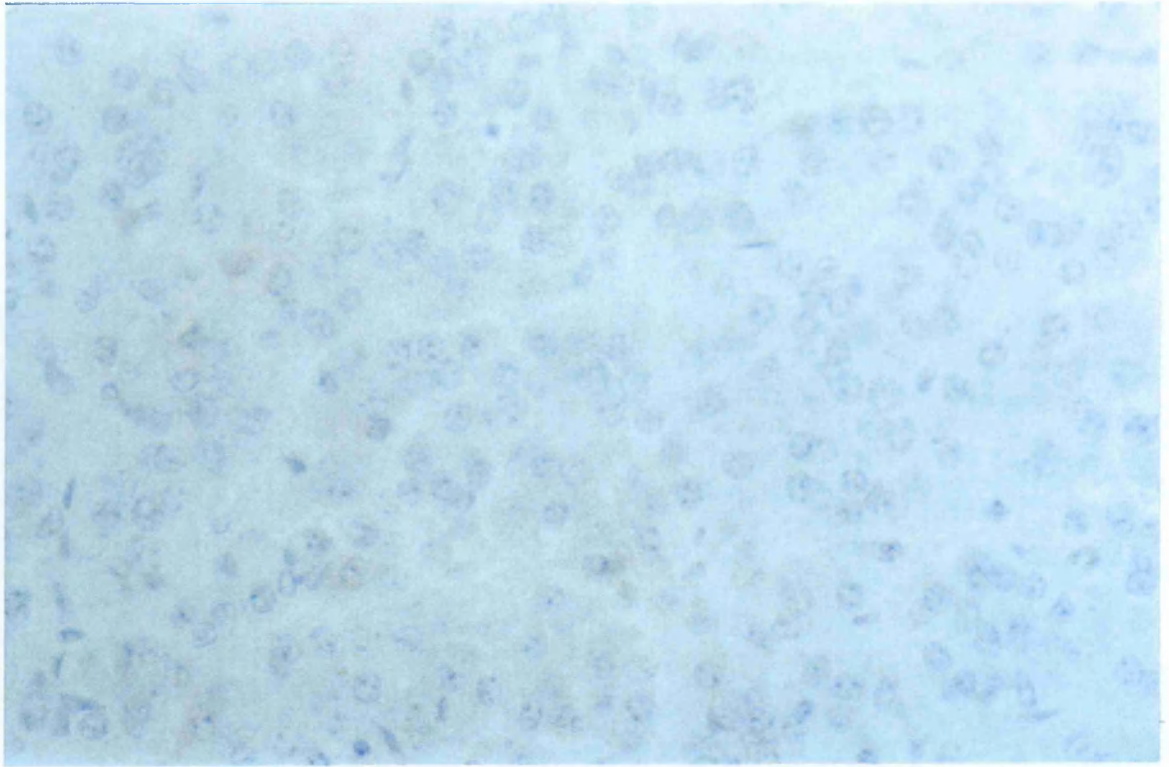
Fig. 5.14. (a) Light microscope pituitary section from lamb 105 (control treatment): ACTH (-) control. ACTH immunoassay, x 40
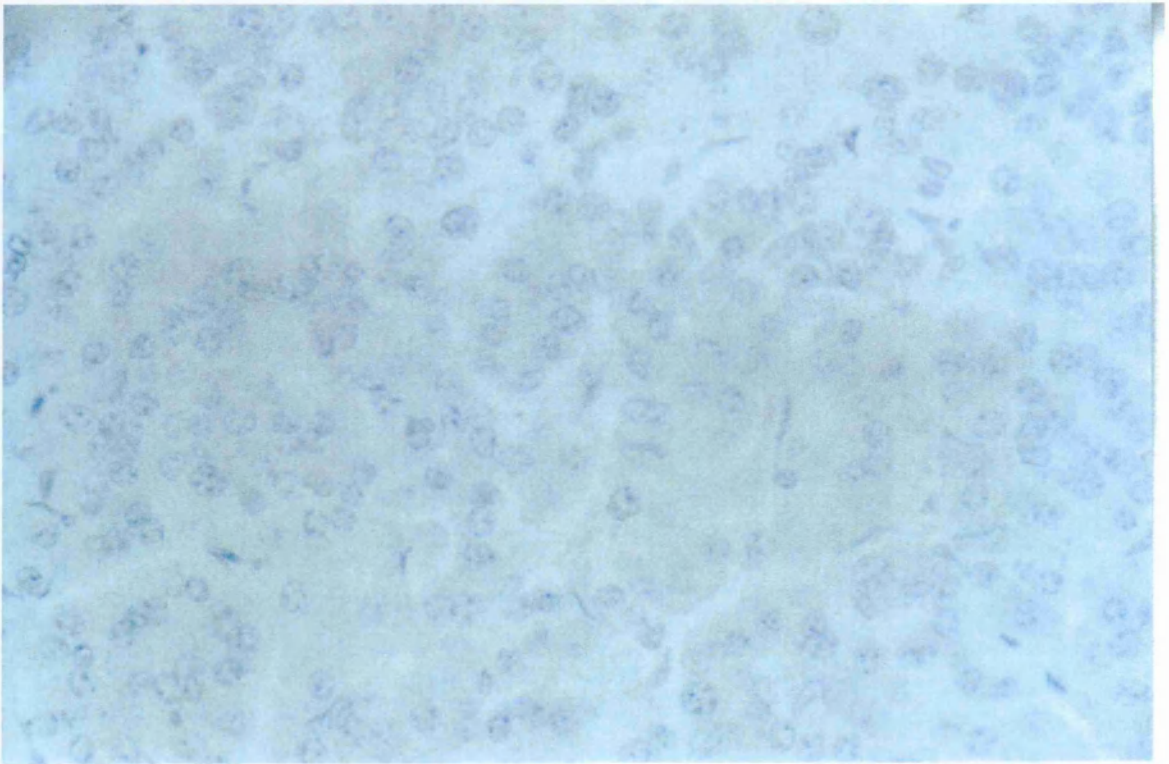
Fig. 5.14. (b) Light microscope pituitary section from lamb 105 (control treatment): ACTH (+) control. ACTH immunoassay, x 40
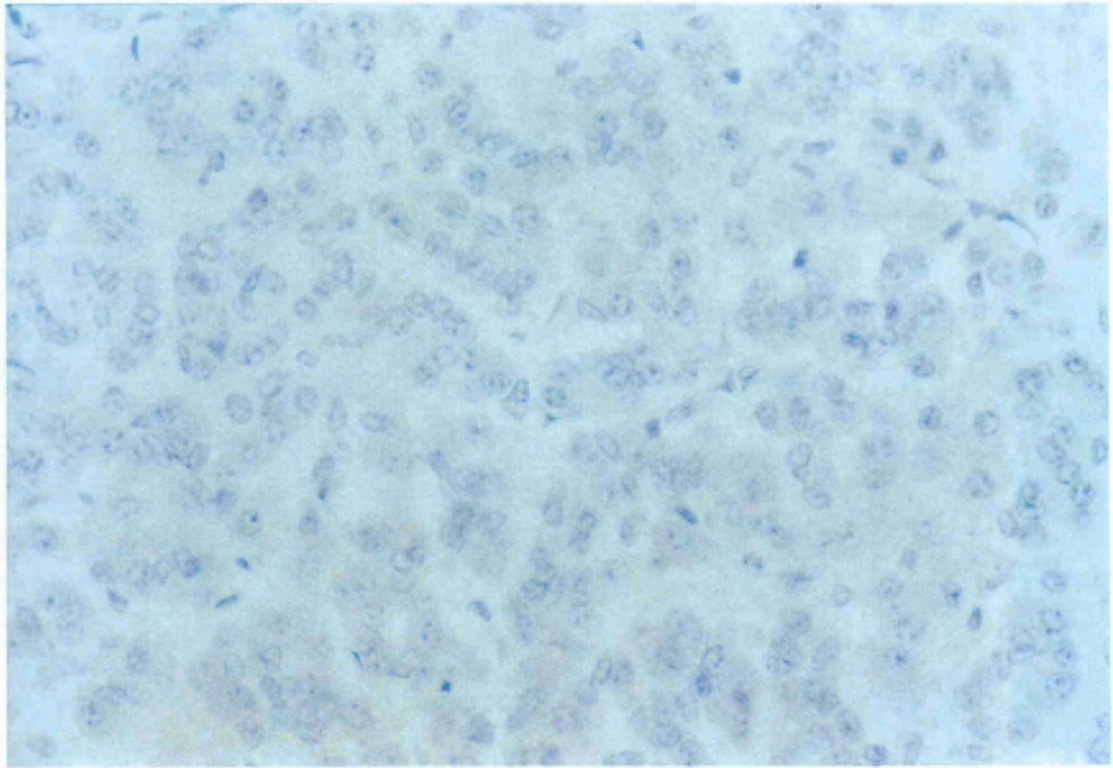
Fig. 5.15. (a) Light microscope pituitary section from lamb 92 (Fe treatment): ACTH (-) control. ACTH immunoassay, x 40
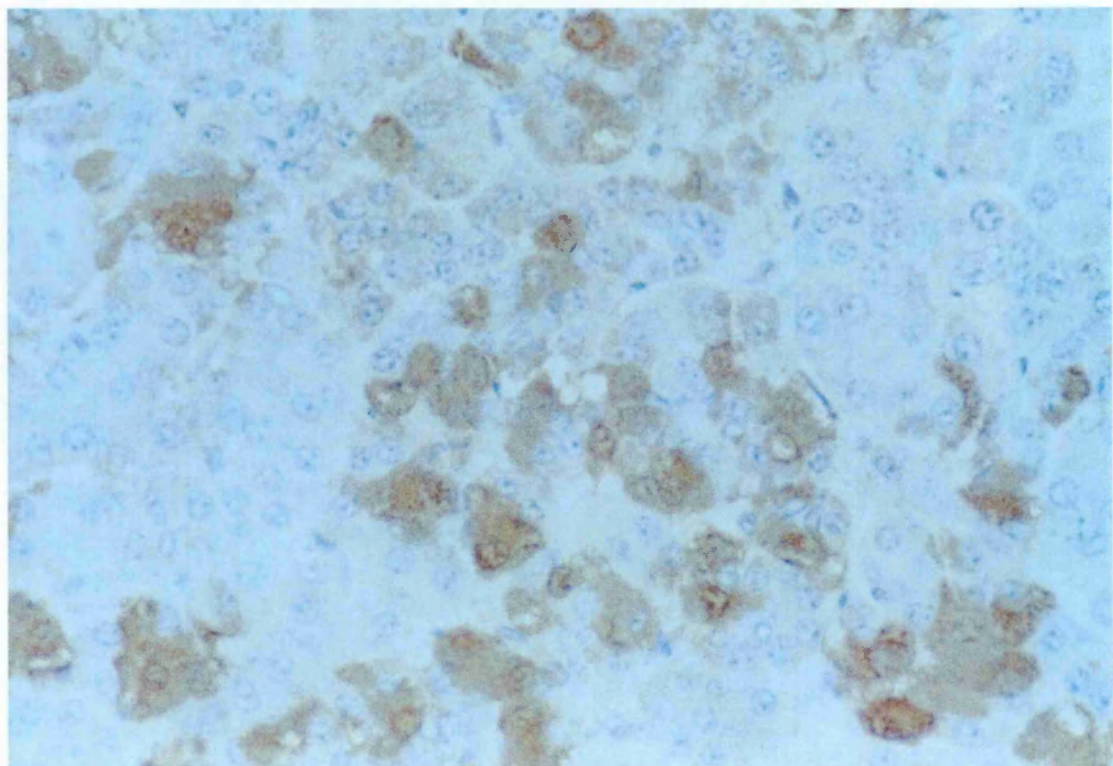
Fig. 5.15. (b) Light microscope pituitary section from lamb 92 (Fe treatment): ACTH (+) control. ACTH immunoassay, x 40
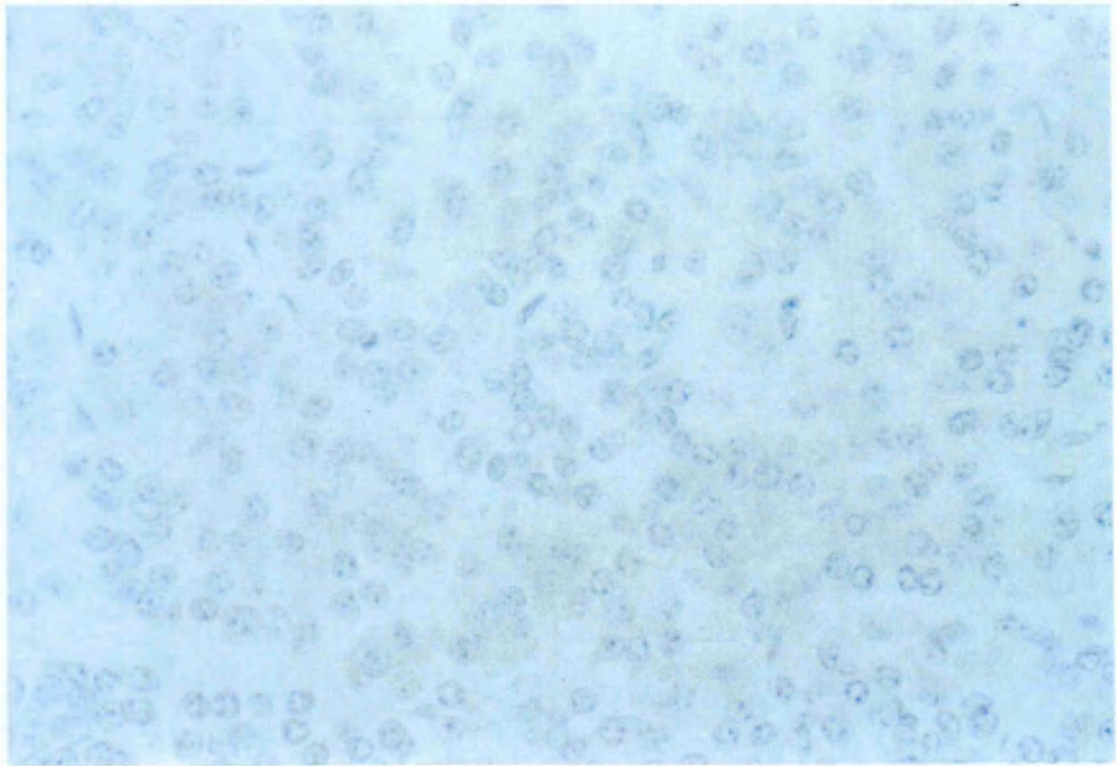
Fig. 5.16. (a) Light microscope pituitary section from lamb 13 (2 Mo treatment):
ACTH (-) control. ACTH immunoassay, x 40
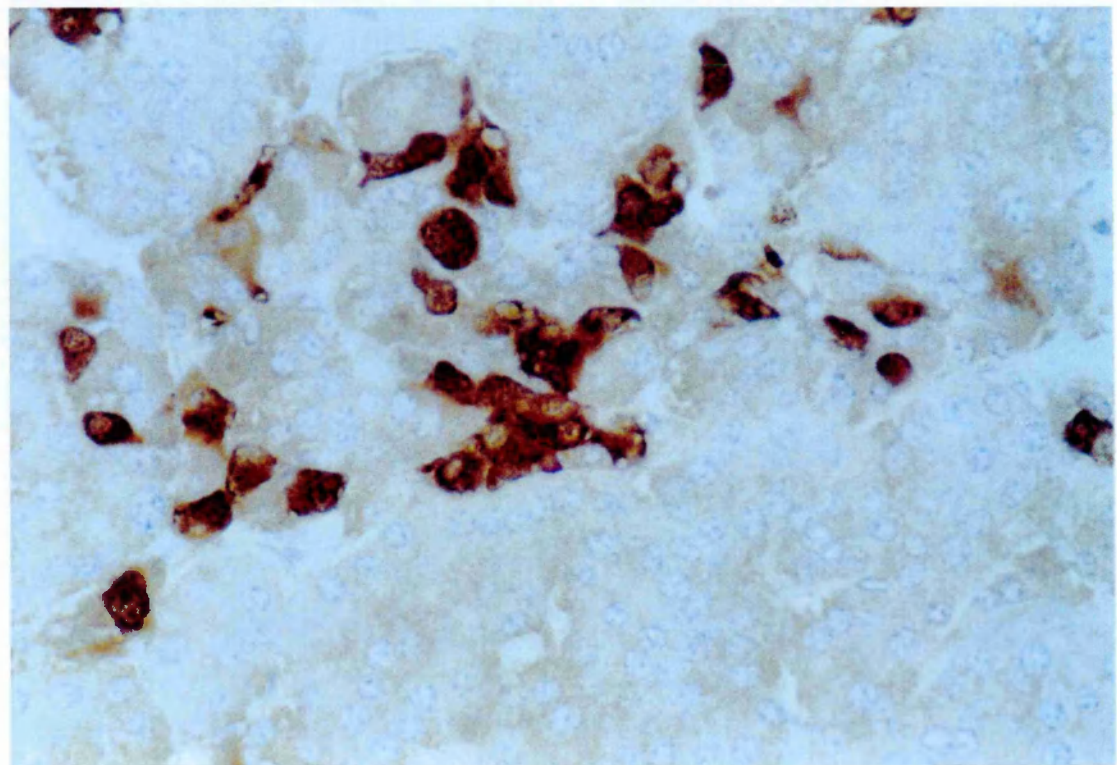
Fig. 5.16 (b) Light microscope pituitary section from lamb 13 (2 Mo treatment):
ACTH (+) control. ACTH immunoassay, x 40
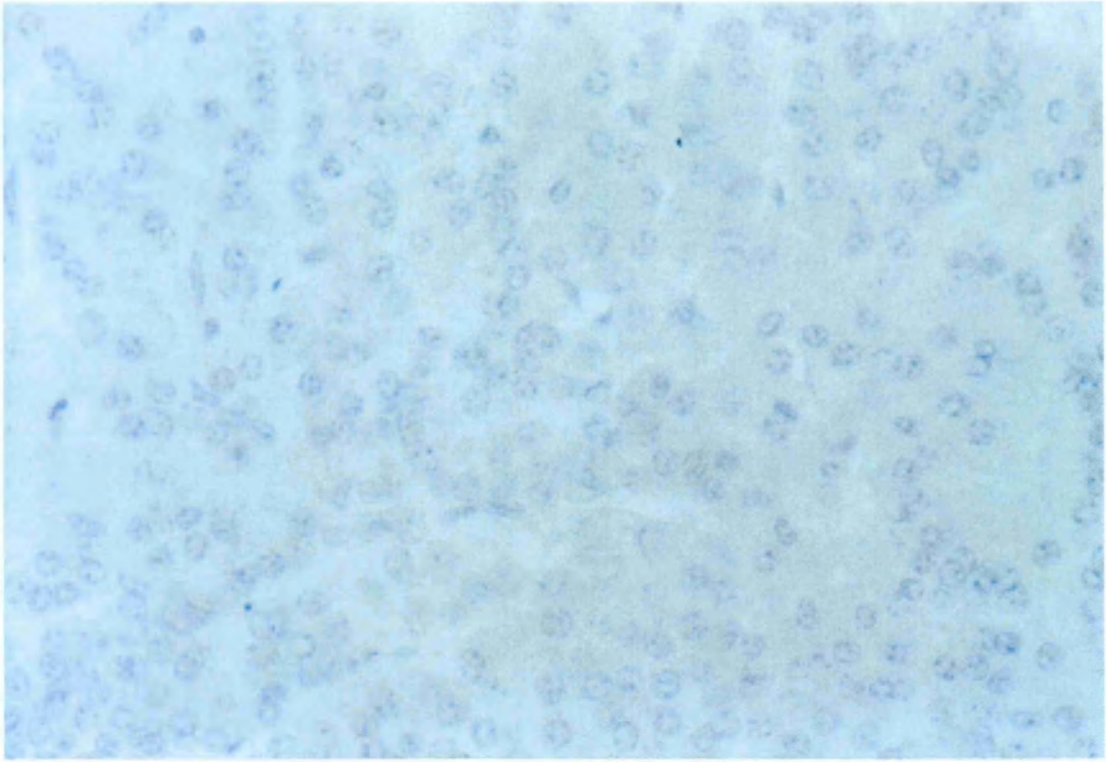
*Fig. 5.17. (a) Light microscope pituitary section from lamb 28 (5 Mo treatment):
ACTH (-) control. ACTH immunoassay, x 40*
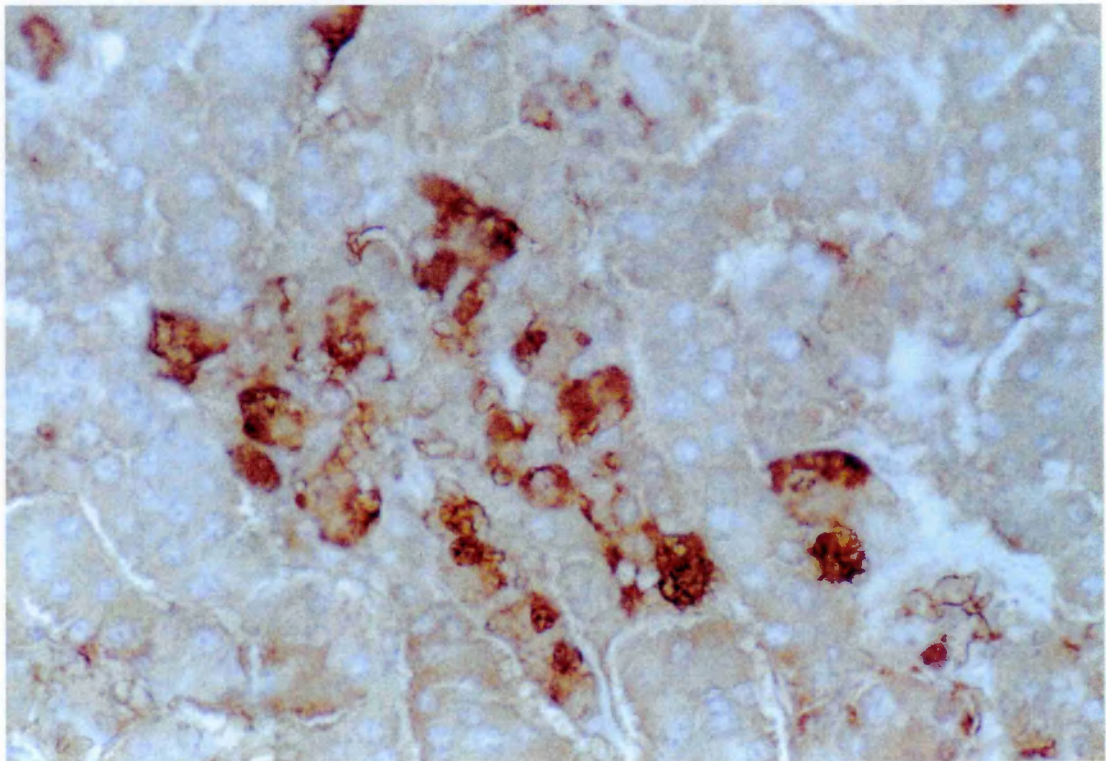
*Fig. 5.17. (b) Light microscope pituitary section from lamb 28 (5 Mo treatment):
ACTH (+) control. ACTH immunoassay, x 40*
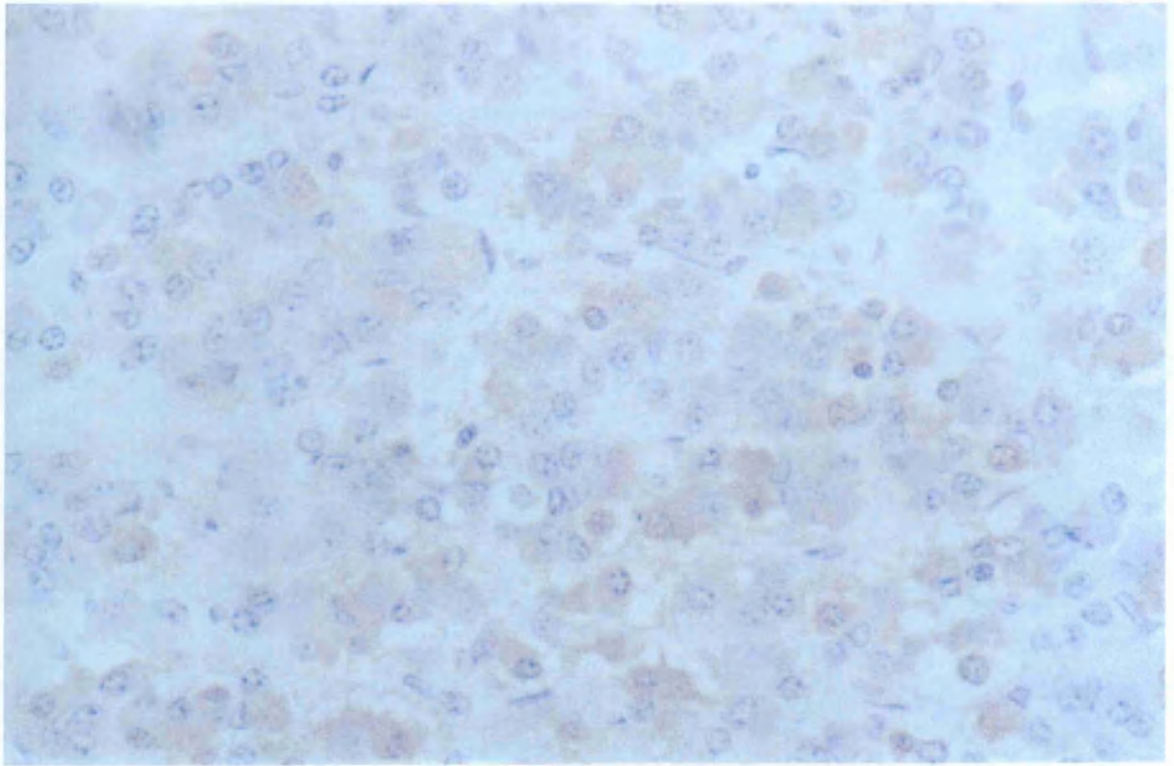
Fig. 5.18.(a) Light microscope pituitary section from lamb 123 (10 Mo treatment): ACTH (-) control. ACTH immunoassay, x 40
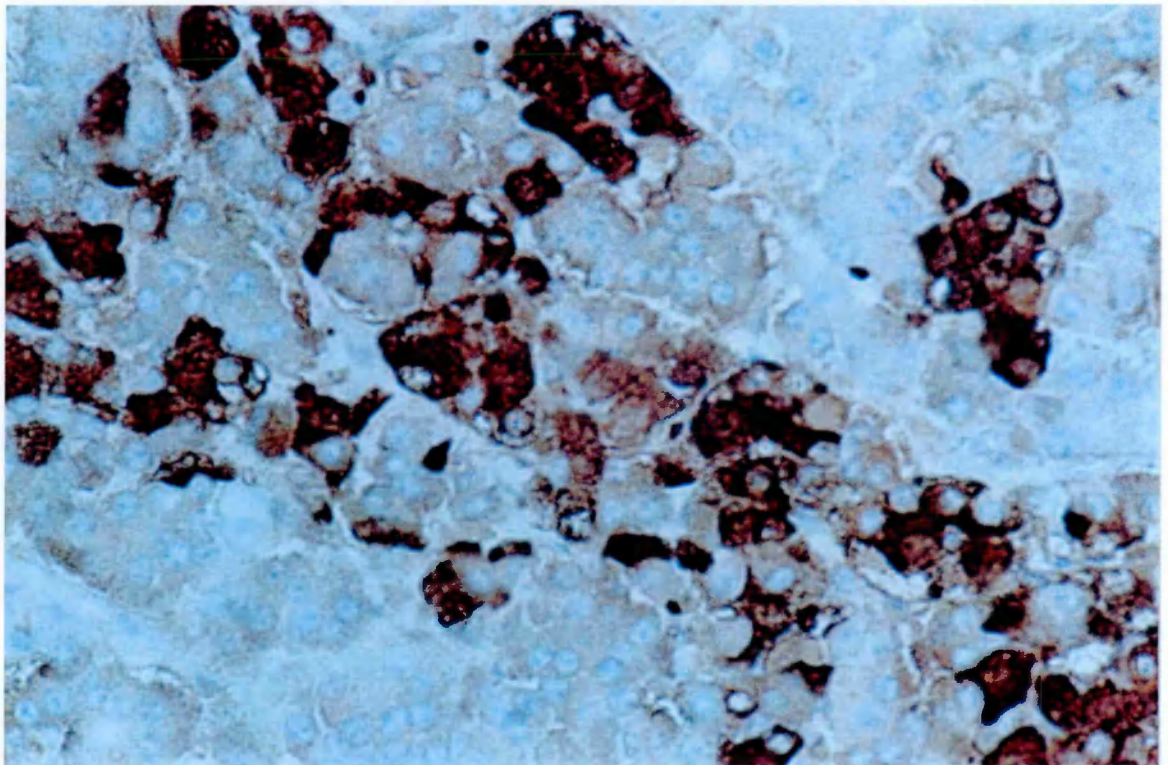
Fig. 5.18.(b) Light microscope pituitary section from lamb 123 (10 Mo treatment): ACTH (+) control. ACTH immunoassay, x 40

154

5.4. Discussion

The aim of this study was to investigate the effects of the Cu antagonists, Mo or Fe, on trace element accumulation in different tissues of growing lambs and to investigate the effects of these Cu antagonists on pituitary and ovarian tissue pathology. Therefore, the Cu parameters used to assess Cu status in this study were similar to those observed in chapters 3 and 4 and will therefore not be discussed in detail. Similar to chapter 3, Pl-Cu concentration was increased by dietary Mo. As in previous chapters, the CP:Pl-Cu ratio was a very useful indicator when assessing Cu status of the animal. The ratio identified lambs that had a lowered Cu status due to dietary Mo as compared to lambs that received the control or Fe dietary treatments. Also in accordance with chapter 3, SOD activity was unaffected by dietary treatment.

Chemical analysis of the control diet was within the normal range for ruminant feedstuffs as specified by ARC (1990). The basal diet was formulated to provide a Cu content of <5 mg kg^{-1} DM, but chemical analysis indicated that the mean Cu content was 6.09 mg kg^{-1} DM. This level was marginally higher than the 1-5.1 mg kg^{-1} Cu DM as recommended by the ARC (1980) for a growing lamb. All other mineral elements were considered to be within the normal ranges for a growing lamb (ARC, 1980).

Normal mean concentration of liver copper in sheep range from 100-500 mg kg^{-1} DM (Underwood, 1977), although sheep are considered to be Cu deficient if their liver Cu concentration is less than 50 µg g^{-1} DM. In this study, the results confirm those obtained in chapter 3 and 4 that dietary Fe and Mo significantly reduced liver Cu concentrations, although only the lambs receiving the 5 mg kg^{-1} Mo dietary treatment would have been considered severely deficient (<50 µg g^{-1} DM). These findings are consistent with those of Ivan and Veira (1985), Van Niekerk and Van Niekerk (1989a) and Bremner and Young (1978). An increase in Pl-Cu concentrations in association with a decrease in liver Cu

155

concentration in lambs that received the 10 mg kg^{-1} dietary treatment may suggest that absorbed TM may cause liver Cu to be released into the plasma as discussed in chapter 3. Liver stores become depleted of Cu as the Cu released becomes bound to circulating TM, resulting in an increased Pl-Cu concentration. It is not clear why the liver Cu concentration of lambs from the 5 Mo group was lower than the 10 Mo group.

Increasing the dietary Mo content of the diet (2, 5 and 10 mg kg^{-1}) resulted in a graduated accumulation of Mo in the liver. These findings are in accordance with Van Niekerk and Van Niekerk (1989a) in ewes and Ivan and Veira (1985) in wethers who concluded that supplementary Mo increased Mo retention within the liver. At present, it is not known if high Mo concentrations in the liver become toxic over time, subsequently causing necrosis and cellular damage. Mo accumulation in livers of Mo supplemented lambs may be stored in an inert form (Van Ryssen and Stielau, 1981; Suttle, 1974b), or may be stored for later use as important components of specific Mo containing enzymes including xanthine oxidase (Abumrad, 1984). There have been no studies investigating the speciation of Mo within the liver or bioavailability of absorbed TM. In this current study, dietary Fe also resulted in significantly higher iron being retained in the liver compared to control and Mo fed lambs. There was no effect of dietary treatment on zinc accumulation in the liver.

In this study, Cu concentration within the ovary of lambs that received 10 Mo was significantly higher than that of the Fe and 2 and 5 Mo dietary treatment groups. Dietary Mo increased Mo concentration in association with an increase in Cu concentration within the ovary. The accumulation of Mo and Cu may have been due to Cu bound TM complexes within the blood that may have been deposited or retained within the ovary. Work by Kendall et al. (2003) found that TTM prevented FSH induced differentiation of bovine ovary granulosa cells in vitro and that TTM altered the morphology of the cultured cells. In addition, TM depressed oestradiol production in a dose dependent manner (Kendall et al., 2003). Impaired reproductive performance in cattle and sheep due to

dietary Mo has previously been reported by other authors (Munro, 1957; Van Niekerk and Van Niekerk, 1989a, b; Phillippo *et al.*, 1982, 1987a, b). Although the precise mechanisms by which high dietary Mo may affect reproductive performance are not fully understood, it is plausible that Mo accumulation in the ovary may affect hormone release and have direct effects on the endocrine control of the pituitary gland. Molybdenum has been found to delay the onset of puberty in heifers (Phillippo *et al.*, 1987b), show low conception rates in dairy cows (Allcroft and Parker, 1949) and show a high incidence of anoestrus in heifers (Munro, 1957; Phillippo *et al.*, 1987b) and ewes (du Plessis *et al.*, 1999a). Phillippo *et al.* (1987b) found that Mo delayed the onset of first oestrus in cattle by at least six weeks and 4-6 weeks in sheep (Van Niekerk and Van Niekerk, 1989b).

Lambs that received the Fe dietary treatment had significantly higher Fe concentrations in the ovary compared to other dietary treatments. The consequence of high Fe concentrations of the ovary is unknown. Phillippo *et al.* (1987b) and Humphries *et al.* (1983) found that an additional 500 mg kg^{-1} dietary Fe to young cattle did not alter reproductive performance to such an extent as 5 mg kg^{-1} Mo. There was no effect of dietary treatment on zinc accumulation in the ovary in this study.

The effect of Mo on ovary histology showed few abnormalities. There was a slightly higher incidence of atretic follicles in the lambs that received the 10 mg kg^{-1} Mo dietary treatment, which may suggest that Mo may cause a greater incidence of follicles to degenerate and prevent maturation due to alterations in the release of LH and FSH from the pituitary. Further research on the direct effects of Mo on hormone accumulation in the pituitary, including LH and FSH, during the reproductive cycle is needed to clarify the exact mechanisms involved in reproductive abnormalities. Phillippo *et al.* (1987b) suggested that the presence of Mo rather than the change in Cu status interferes with the events that control the oestrous cycles, causing a decrease in LH release and altered ovarian steroid secretion.

The pituitary gland, and the specific trophic hormones that it secretes, regulates processes including growth, metabolism, protein synthesis and ovarian function. These hormones include luteinising hormone (LH), oestrogen, thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH) and progesterone. Therefore, the decrease in production or expression of hormones involved with reproductive function and growth in ruminants may be altered due to high Mo intakes. For the onset of puberty to occur and for the first ovulation to take place, a surge in LH is required (Moffor and Rodway, 1991). Phillippo *et al.* (1987b) and du Plessis *et al.* (1999a) showed that the secretion of LH was reduced in animals that received dietary Mo. The neuroendocrine regulation and release of hormones from the anterior pituitary gland may be directly linked to activities within the ovary that may be directly affected by dietary antagonists.

Adrenocorticotrophic hormone (ACTH) is released from the anterior pituitary gland and then acts upon the adrenal glands. Within the pituitary, the cuproenzyme, peptidylglycine α-amidating monooxygensase (PAM) is a bifunctional protein containing PHM (peptidyglycine α-hydroxylating mono-oxygenase) (Bradbury *et al.*, 1982) and PAL (peptidylamido-glycolate lyase) (Katopodis *et al.*, 1990), both essential in a two step conversion of a peptidylglycine substrate into an amidated product. Pituitary cells express high levels of PAM and store PAM and its amidating products in secretory granules (Braas *et al.*, 1989; Eipper *et al.*, 1993) but require Cu for effective functioning and release of pituitary hormones. Findings from this study reveal that the effects of dietary Mo and the possible formation of TM, may reduce the Cu that is bound to PHM resulting in accumulation of hormones or their releasing factors in the cells of the pituitary gland. Lambs that received the dietary Mo in this study had obvious accumulation of ACTH within the pituitary cells compared to other dietary treatment groups, possibly as a direct result of the failure of PAM to release the hormone. Although ACTH was the only hormone studied, a similar mechanism for hormone release may occur for other hormones

released from the anterior pituitary gland, including LH and FSH. The failure to secrete hormones (or the respective trophic hormones) may consequently alter adrenal and ovarian activity. A reduction in PAM activity may also account for the lower LH release as seen in lambs receiving intravenous TTM (Moffor and Rodway, 1991) as it is known that PAM is regulated by oestrogen status (El Meskini *et al.*, 1998). Phillippo *et al.* (1987a) reported a significant reduction in pulsatile release of LH, subsequently delaying puberty, reducing conception rates and with an increased anoestrous activity in young cattle due to supplementation of 5 mg kg^{-1} Mo. Van Niekerk and Van Niekerk (1989b) suggested that Mo and S may reduce the LH and FSH concentrations and also lower receptor sensitivity in the ovary. Wise and Ferrell (1984) found that heifers supplemented with Mo had such a severe reduction in LH ovulatory peaks that exogenous LH severely affected conception rates. Dietary Mo therefore mediates its effects on ovarian steroid morphology and production as well as the hypothalamo-pituitary axis with a possible direct effect on PAM in the release of pituitary hormones.

Work by Haywood *et al.* (1998) found that TTM given parentally to different breeds of sheep resulted in Mo accumulation in the brain, adrenal glands, heart and gonads. In a later report, Haywood *et al.* (2004), it was noted that due to tetrathiomolybdate administration, pituitaries were small and mis-shapen due to diminuation of the anterior lobe. In this current study, immunocytochemistry indicated that affected cells of the pituitary from lambs that received Mo seemed to contain swollen cells and darkened mitochondria, which were similar to those observed by Van Ryssen *et al.* (1982) in the kidney of sheep that were fed high dietary levels of Mo. Mo or TM may therefore alter the morphology of pituitary cells types although this was not assessed in this study.

Within the brain, Cu, Fe and Zn play an important role as components of proteins essential for neural functioning (Armstrong *et al.* 2001). In this study, there was no effect of dietary

treatment on Cu, Mo, Zn or Fe accumulation in the cerebellum. These findings are in contrast to Haywood *et al.* (1998) who found that when Mo is given parentally as TTM (irrespective of Cu status), Mo is selectively retained in all parts of the brain (hypothalamus, cerebellum, cerebrum and medulla oblongata). It was suggested by Haywood *et al.* (1998) that TM may circulate within the bloodstream and cross the blood-brain barrier, where Cu or Mo is deposited within areas of the brain. A later report by Haywood *et al.* (2004) similarly found that ammonium tetrathiomolybdate given to Cu poisoned sheep caused Cu and Mo content to be raised in all areas of the brain. Therefore, the same scenario may occur due to an increased or longer Mo intake in sheep and cattle. More research is required to identify if Cu-TM may permeate the blood-brain barrier and have localised toxic effects on cells and tissues.

Decreased or increased Cu availability has been implicated to be a causative factor of several other neurodegenerative diseases in man and animals, associated with the overproduction of reactive oxygen species (ROS) (Sies, 1993). These diseases include prion (PrP^c) diseases, Alzheimers disease (AD) and Parkinsons disease (PD) (Jellinger, 1999). Normal prion proteins are Cu dependent proteins which protect the cell against oxidative damage (Brown *et al.* 1999), whereas abnormal apoglycoproteins (PrP^{sc}) lack Cu and cause severe neurodegenerative disorders in animals and man (Brown *et al.*, 1997). Conversely, AD, is exacerbated by marked elevation of brain Cu and Fe which disrupts the β-Amyloid cuproprotein (Bush, 2000) and causes deposition of β-amyloid within the neocortex (Lovell *et al.*, 1998). The pathology of PD has also been suggested to be caused by high levels of Cu and Fe causing the subsequent overproduction of free radicals and death of nigral cells (Jellinger, 1999). An abnormal pathogenic apoglycoprotein (PrP^{SC}) which lacks Cu has been suggested to be related to animal diseases including sheep scrapie, bovine spongiform encephalopathy (BSE) and transmissible mink encephalopathy (TME) (Sorenson, 2001). Therefore, the relationship between these diseases and an altered Cu metabolism suggest an important role of metals in neurological diseases and the related

tissue-specific effects as seen in this study. Current findings may be critical in helping unravel the pathogenesis of a Mo-induced Cu deficiency in the future.

5.5. Conclusion

In conclusion, this study identified that dietary Mo altered Cu metabolism in growing lambs as measured by their Cu status. In addition to alterations in blood constituents, dietary Mo was found to cause Mo to accumulate in the liver in association with a significant reduction in liver Cu concentration. Mo also accumulated in the ovary of Mo supplemented lambs. Dietary Fe caused accumulation of Fe in the liver and ovary. Mineral retention in the cerebellum and pituitary gland was not affected by dietary treatment. Histopathology of the ovary revealed an increase in the number of atretic follicles from Mo supplemented lambs. Pathology of the pituitary gland indicated that ACTH accumulated in cells from Mo supplemented lambs, possibly due to the inhibition of the cuproenzyme PAM which prevented the release of this hormone effectively from the anterior pituitary gland.

Chapter 6

The effect of molybdenum or iron on copper status and ceruloplasmin expression in the liver of growing lambs

6.1. Introduction

Results obtained in previous chapters have demonstrated that dietary Mo reduces ceruloplasmin (CP) activity but the mechanism by which this occurs remains unclear. As discussed previously, a reduction in CP activity may either be attributable to the effect of TM directly on CP within the blood or may be due to a direct effect of TM within the hepatocyte, either by altering the ability of the Cu chaperones to distribute Cu effectively to CP or by altering the expression of CP directly.

Under normal conditions, CP is synthesised by the hepatocytes in the liver and is secreted into the plasma as holo-CP with 6-7 atoms of tightly bound Cu (Lockhart and Mercer, 1999). CP is known to be produced at a constitutive rate in the liver as shown using dot-blot mRNA quantification in Cu sufficient and Cu deficient rats (Gitlin *et al.*, 1992) but it is not known if TM may alter the expression of the protein. CP activity may also be reduced as a consequence of systemic TM affecting the Cu chaperone ATOX1 (the Cu chaperone involved in delivering Cu to the apo-CP protein).

Bissig *et al.* (2001) demonstrated that the gram-positive bacteria *Enterococcus hirae*, encoded two types of Cu P-type ATPases, CopA and CopB. These ATPases were found to act as intracellular Cu 'pumps' but were found to be inhibited by di, tri and tetra-TM but this inhibition could be reversed by Cu or silver. Mason *et al.* (1982) found that both duodenal and intravenous infusions of tri and tetra TM produced rapid decreases in CP activity in sheep, but TM were reversible inhibitors of CP activity *in vitro*.

Therefore, the aim of this study was to investigate the effects of Mo and Fe on hepatic CP mRNA expression and Cu status of Scottish Blackface wethers and to investigate whether

the differences in CP activity as previously reported in chapters 3, 4 and 5 were due to alterations in gene expression of CP.

164

6.2. Materials and methods

6.2.1. Experimental Design and Animals

Thirty male Scottish Blackface wethers, approximately 14 months old, with an initial mean live weight of 35.1 kg (s.e.d. 1.54) were used in this study. All wethers were allocated by live weight to one of three dietary treatment groups (section 6.2.2.) for a period of twelve weeks. All wethers were individually penned on elevated metal floors in a ventilated barn throughout the experimental period. Prior to the start of the trial, wethers were fed the basal control diet at 1 kg per day plus hay *ad libitum*. Hay intake was decreased to zero before the trial period began. By day 0 of the trial, all wethers were receiving only the control diet. At day 0, all wethers received their allocated treatment diet (see section 6.2.2.).

6.2.2. Diet formulation

The raw materials used for this study were chosen because of their published low Cu and Mo contents (MAFF, 1992). Based on this information (Table 6.1.), the diets formulated were predicted to provide a Cu content of <5 mg kg^{-1} DM. The basal diet was formulated to provide the metabolisable energy (ME) and metabolisable protein (MP) requirements of a male sheep growing at 150g day^{-1} (AFRC, 1993) and had a predicted ERDP of 99.84 g kg^{-1} DM and an FME of 9.885 MJ kg^{-1} DM.

Lambs were allocated by live weight to one of three dietary treatment groups which were:

Treatment one: Basal diet (Control)

Treatment two: Basal diet + 500 mg kg^{-1} DM iron and 2 g kg^{-1} DM sulphur (Fe)

Treatment three: Basal diet + 5 mg kg^{-1} DM molybdenum and 2 g kg^{-1} DM sulphur (5 Mo)

165

Feed samples were obtained from separate tote bags once weekly. All feed samples were analysed for dry matter, crude protein, neutral detergent fibre, ether extract, ash and neutral cellulase gamanase digestibility (NCGD) as described in sections 2.1.1. to 2.1.6 respectively. The raw material and chemical composition of the basal diet is presented in Table 6.1. Additional mineral inclusion per treatment diet is presented in Table 6.2.

Table 6.1. Diet formulation and analysed chemical composition of the basal diet (DM)

Ingredient (g/kg^{-1})	Basal Diet
Straw pellets (NaOH treated)	400
Ground maize	250
Rapeseed meal	270
Molasses	45
Mineral/vitamin premix[1]	35
Total	1000
Dry matter (g kg^{-1} DM)	872.6
ME (MJ kg^{-1} DM)	10.7*
Crude protein (g kg^{-1} DM)	153.7
Neutral detergent fibre (g kg^{-1} DM)	443.9
Ether extract (g kg^{-1} DM)	21.6
Ash (g kg^{-1} DM)	107.3
NCDG (g kg^{-1} DM)	732.3

* Predicted values from equations given in AFRC (1993)
[1] Mineral premix – see Table 6.2

Table 6.2   Additional mineral inclusion for the control, iron and molybdenum treatment diets (kg ton^{-1})

Ingredient	Treatment diet		
	Control	Fe	5 Mo
Ammonium molybdate (kg)	-	-	0.008
Iron sulphate (kg)	-	2.15	-
Ammonium sulphate (kg)	-	6.06	7.078
Urea[2]	3.2	0.46	-
Sand (kg)	6.8	1.33	2.914
Mineral Premix[1]	25	25	25
Total (kg)	35	35	35

[1]Mineral premix (25kg per ton) commercial lamb vitamin/mineral premix (S.C. Feeds Ltd, Stone, UK) containing 250,000 IU/g Vit A ; 50,000 IU/g Vit D3 ; 4,000 mg kg^{-1} Vit. E ; 15 mg kg^{-1} selenium ; iodine mg kg^{-1} ; cobalt 60mg kg^{-1} ; manganese 2,000 mg kg^{-1}; zinc 2,000 mg kg^{-1}; phosphorus 2% ; magnesium 5% and salt 20.2%.
[2]Urea (Trouw Nutrition, Northwich, Cheshire, UK)

The mineral composition of the basal diet was determined by ICP-ES (see section 2.3) and is presented in Table 6.3. Molybdenum was supplemented as reagent grade ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4\ H_2O$) , iron was supplemented as reagent grade iron sulphate ($FeSO_4.7H_2O$) and sulphur was supplemented as reagent grade ammonium sulphate ($(NH_4)_2SO_4$) (all BDH Laboratory Supplies, Poole, Dorset, UK). The nitrogen content of the mineral mix was balanced with urea (Trouw Nutrition, Northwich, Cheshire, UK) and sand was used as an inert material to balance mass.

Table 6.3. Analysed mineral composition of basal diet

Element	Content
Calcium (g kg^{-1})	9.90
Phosphorus (g kg^{-1})	4.10
Sulphur (g kg^{-1})	2.80
Magnesium (mg kg^{-1})	3.30
Copper (mg kg^{-1})	5.50
Molybdenum (mg kg^{-1})	0.67
Iron (mg kg^{-1})	206.69
Zinc (mg kg^{-1})	57.44
Cobalt (mg kg^{-1})	1.41
Selenium (mg kg^{-1})	0.40
Manganese (mg kg^{-1})	72.19

Water was offered *ad libitum* and the copper content of the water was 35µg/l with no detectable molybdenum (Dee Valley Group Laboratory, Rhostyllen, Wrexham, UK).

6.2.3. Experimental Routine

All wethers were fed at 08:00 and 15:00 daily. Feed was weighed out into individually designated clean plastic buckets to prevent mineral contamination, weighed using metric scales (F.J. Thornton and Co. Ltd., Wolverhampton, UK) and calibrated using metric standard weights. Food refusals were weighed back twice weekly to determine individual lamb food intakes. The quantity of the feed offered was recalculated weekly according to the live weight of the animal recorded on the day of live weight determination (see section 6.2.4.) in order to achieve a growth rate of 150 g day^{-1}.

6.2.3.1. Liveweight determination

All wethers were weighed once weekly on Mondays at 11:00 using the standard operating procedure as described in section 2.3. Daily live weight gain was calculated using regression analysis.

6.2.3.2. Blood collection and analysis

Blood samples were collected fortnightly from all wethers on Tuesdays at 10:00 as described in section 2.2. Fresh whole blood was used to assess haematology profile (section 6.2.3.3.), and the remaining blood prepared (serum or plasma) as described in section 2.2 and stored at $-20°C$ for subsequent analysis of ceruloplasmin (CP) (section 2.2.4.), superoxide dismutase (SOD) (section 2.2.5.), plasma copper (Pl-Cu) (section 2.2.6.) and amine oxidase (AMOX) (section 2.2.7). The intra assay coefficient of variation for respective standards were: CP 3.42, SOD 3.58, Pl-Cu 6.17 and AMOX 2.43.

6.2.3.3. Haematology profile

Blood samples were collected from all wethers into EDTA vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK). A haematology profile was obtained using an automated Scil Veterinary ABC animal blood counter (ABX Diagnostics, Shefford, Bedfordshire, UK). A MINOTROL™ 16 blood control (ABX Diagnostics, Shefford, Bedfordshire, UK) was used before every batch of fresh blood samples. Blood was obtained and analysed for total white blood cell concentration (WBC), red blood cell concentration (RBC), haemoglobin concentration (Hb), haematocrit (Hc), platelet concentration (PLT), mean corpuscular volume (MCV), mean cell haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

6.2.4. Liver collection procedure

All wethers were sent to a commercial slaughterhouse after 13 weeks of the trial. All wethers were electrically stunned and killed by exsanguination. For each wether, a small incision (7 cm) was made through the skin at a site below the sternum to obtain a small sections of liver (approximately 100 g) within two minutes from being bled. A sterile liver biopsy needle (Cook Inc., Bloomington, IN, USA) was used to obtain five small samples of liver (approximately 20-30 mg each) from the 100 g sample, which were then placed into 1.5ml RNA-ase free micro-centrifuge tubes and placed immediately on dry ice at -80°C for subsequent extraction of the RNA (see section 6.2.4.). Sterile latex gloves were used for all procedures to prevent RNA and DNA contamination to the samples. The remaining whole liver was removed at a later stage, stored in a clean plastic bag and frozen at -20°C for subsequent trace element determination using ICP-MS as described in (section 2.2.9.).

6.2.5. mRNA and DNA extraction

Total cellular mRNA and DNA was extracted using a SV Total RNA Isolation System kit and Wizard® Genomic DNA purification kit respectively (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and quality of the RNA and DNA was assessed spectrophotometrically (Beckman DU640, Fullerton, California) with the ratio of A260nm/A230nm (RNA only) and A260nm/A280nm being within the expected ranges (1.8-2.2 and 1.7-2.1 respectively). DNA was stored at 4°C and RNA was stored at –80°C until required.

6.2.5.1. Primer design

Primers were designed using Primer 3 software (http://workbench.sdsc.edu/) for the sheep ceruloplasmin (CP) mRNA from conserved regions which flanked intron sites within the following sequences: sheep mRNA (EMBL accession no. AF134841), mouse mRNA

170

(EMBL accession no. MM49430), rat mRNA (EMBL accession no. RNCERU) and rat DNA exon 1 (accession no. RNCERP) after sequence alignment using CLUSTAL W (Thompson *et al.*, 1994). β-Actin primers were designed from sheep mRNA (EMBL accession no. AFO35422), rat mRNA (EMBL accession no. RNAC01) and pig DNA (EMBL SSC312193) as described above for the ceruloplasmin gene. Primers were designed to be approximimately 20 base pairs (bp) long with a melting temperature of approximately 60°C.

6.2.5.2. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the RT-PCR System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. A ten-fold dilution of extracted RNA (approximately 20 ng/μl) and DNA (approximately 20 ng/μl) were amplified (5μl in 25μl volume). RNA samples from randomly chosen wethers (ear-tag no. 596 and 704) and DNA samples from wethers (ear-tag 313 and 538) were amplified using the following primer pairs: (CPF1/CPR1, CPF2/CPR2, baF1/baR1, baF2/baR2).

Table 6.4. Primer design, melting temperature and product size of sequences used for RT-PCR

Primer Name	Sequence	Product Size (DNA)	Estimated Product Size (cDNA)
CPF1	TCTGGGCGAAAGATAAGCAT		
CPR1	AATGGTAAATCCGGGTCACA	{ 492 + intron	492
CPF2	GTACCTCAGTCTGGGCGAAA		
CPR2	TAAAGGCCCAATGACTCCTG	{ 551+ intron	551
baF1	TCCCTGGAGAAGAGCTACGA		
baR1	GGGCAGTGATTTCTTTCTGC	{ 350	255
baF2	GAAATCGTCCGTGACATCAA		
baR2	AGCACCGTGTTGGGATAGAG	{ 362	267

RNA samples were amplified according to the manufacturer's instructions except anneal temperatures of 55°C, 58°C and 60°C were tested to determine suitable primers and optimum RT-PCR conditions. All RNA samples were then amplified in a RT-PCR using primers CPF1/R1 and baF1/R1 at an anneal temperature of 60°C. DNA samples were amplified under the same conditions as detailed by Edwards *et al.* (2001) using the same PCR programme as for RNA. PCR products were separated on 2% agarose gel amended with 0.05% ethidium bromide by electrophoresis and analysed using a GelDoc 1000 gel documentation system (Biorad, Hemel Hempstead, UK). Band intensity was measured on an unsaturated image using Molecular Analyst (Biorad) and a ratio of CP to β-actin (ba) mRNA product was determined. Control amplifications included a negative control, a positive control supplied with the RT-PCR kit and a pure and 10-fold dilution of RNA from wether no. 706 which was known to amplify during preliminary experiments.

6.2.6. Statistical Analysis

Statistical analysis was performed using Genstat for Windows (Version 5.0). All haematology analysis, enzyme activities, liver trace element concentration and mRNA ratio was analysed by analysis of variance (ANOVA) and repeated measures (where appropriate) using a completely randomized design. Daily liveweight gain (DLWG) was determined by regression analysis. In all graphs, error bars refer to 2x s.e.d. Differences between treatments was calculated using a protected least significant difference (LSD) (Snedecor and Cochran, 1989).

6.3. Results

6.3.1. Growth rate and performance characteristics

There was no significant effect of dietary treatement on wether live weight at any weekly timepoint (Fig. 6.1). There was no significant effect of dietary treatment on total gain, daily live weight gain (DLWG), daily dry matter intake (daily DMI), total dry matter intake or food conversion efficiency (FCE) (Table 6.4). The mean DLWG for all treatments was 151.7 g day^{-1}, which was similar to the predicted 150 g day^{-1} as predicted by AFRC (1993) (Table 6.5).

Table 6.5. *Effect of molybdenum or iron supplementation on food intake and performance of Scottish Blackface wethers (kg)*

	Treatment diet				
	Control	Fe	5 Mo	s.e.d.	Significance
Initial wt	33.2	32.8	33.1	1.11	NS
Slaughter wt	46.2	46.8	46.1	1.77	NS
Total Gain	13.04	14.06	13.08	1.034	NS
DLWG	0.148	0.159	0.148	0.1197	NS
Daily DMI	1.42	1.43	1.43	0.043	NS
Total DMI	119.3	120.5	119.9	3.57	NS
FCE*	0.109	0.117	0.109	0.0073	NS

* FCE calculated as Total Gain (kg) divided by Total DMI (kg)
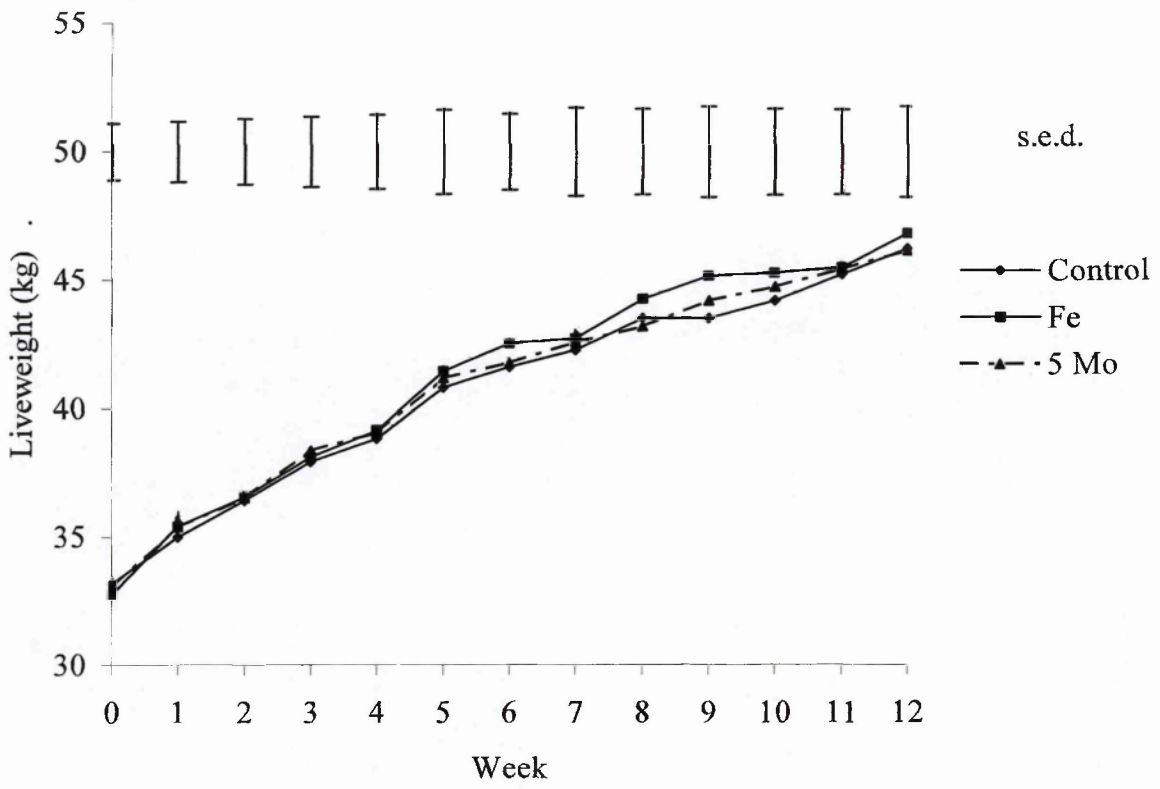
Fig 6.1.   *Effect of molybdenum or iron supplementation on live weight of Scottish Blackface wethers*

6.3.2. Haematology profile

All components of the haematology profile were within normal expected ranges for sheep (see Appendix 1).

6.3.2.1. Haematocrit (Hc)

There was no significant effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on Hc of wethers (Table 6.6.). Repeated measures analysis indicated that there was an effect (P<0.001) of time due to the decrease in Hc from week 0 to week 12 in all treatment groups, but no effect of treatment and no time x treatment interaction on Hc.

Table 6.6.   *Effect of molybdenum or iron supplementation on haematocrit (%) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	37.05	39.40	38.54	1.393	NS
2	36.07	37.30	36.34	1.822	NS
4	32.95	32.56	34.56	1.789	NS
6	33.70	34.60	34.55	1.502	NS
8	31.90	32.99	34.33	1.633	NS
10	34.20	34.43	35.82	1.706	NS
12	33.93	35.22	35.25	1.675	NS

Repeated Measures Analysis:	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

6.3.2.2. Haemoglobin (Hb) concentration

There was no significant effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on Hb concentration

(g/100 ml) (Table 6.7.). Repeated measures analysis indicated that there was an effect

(P<0.001) of time on Hb concentration possibly due to the small decrease in all treatment

groups over time but no effect of treatment or time x treatment interaction on Hb

concentration.

Table 6.7. *Effect of molybdenum or iron supplementation on haemoglobin (Hb) concentration (g/100 ml) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	12.64	12.98	12.58	0.382	NS
2	12.98	12.92	12.76	0.432	NS
4	12.25	11.91	12.26	0.534	NS
6	12.14	12.25	11.87	0.383	NS
8	11.48	11.63	11.60	1.663	NS
10	12.23	12.10	12.32	0.434	NS
12	12.30	12.48	12.16	0.485	NS

Repeated Measures Analysis: Treatment effect   P = NS

Time effect   P = <0.001

Time X Treatment effect   P = NS

6.3.2.3. Red Blood Cell Count (RBC)

There was no significant effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on RBC concentration (cells x 10^{12} l^{-1}) of wethers (Table 6.8.). Repeated measures analysis indicated that there was an effect (P<0.001) of time due to the decrease in RBC concentration in all treatment groups from week 0 to week 12. There was no effect of treatment or time X treatment interaction on RBC concentration.

Table 6.8. *Effect of molybdenum or iron supplementation on red blood cell (RBC) concentration (cells x 10^{12} l^{-1}) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	12.98	13.62	12.88	0.440	NS
2	12.39	12.65	11.92	0.509	NS
4	11.35	11.07	11.39	0.532	NS
6	11.59	11.77	11.39	0.404	NS
8	10.98	11.19	11.28	0.496	NS
10	11.53	11.36	11.43	0.450	NS
12	11.33	11.43	11.11	0.477	NS

Repeated Measures Analysis:

Treatment effect	P = NS
Time effect	P = <0.001
Time X Treatment effect	P = NS

6.3.2.4. Mean corpuscular volume (MCV)

There was no effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on MCV of wethers until week 12 (Table 6.9.). At week 12, lambs that received 5 mg kg^{-1} Mo had a higher MCV (P<0.05) compared to the wethers that received the control or 500 mg kg^{-1} Fe dietary treatments. Repeated measures analysis indicated that there was an effect (P<0.001) of time with all treatment groups having an increase in MCV from week 2 until week 12. There was no effect of treatment or time X treatment interaction on MCV.

Table 6.9.   *Effect of molybdenum or iron supplementation on mean corpuscular volume (MCV) (fl) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
2	29.8	29.7	29.4	0.24	NS
4	29.5	29.5	29.4	0.24	NS
6	29.5	29.6	29.8	0.28	NS
8	29.4	29.6	29.8	0.34	NS
10	30.0	30.5	30.7	0.42	NS
12	30.0[b]	30.1[b]	31.1[a]	0.40	*

[a,b] Means within a row with different superscripts are significantly different
* <0.05, ** <0.01, *** <0.001
Mean corpuscular volume (fl) (MCV) = (Haematocrit % / RBC x 10^{12}/l) x 10 (Kerr, 2002)

Repeated Measures Analysis: Treatment effect   P = NS

                                 Time effect   P = <0.001

                                   Time X Treatment effect   P = NS

6.3.2.5. Mean Cell Haemoglobin (MCH)

There was no significant effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo *on* MCH concentration (pg) (Table 6.10.). Repeated measures analysis indicated that there was an effect of time (P<0.001) with all treatment groups having an increased MCH from week 0 to week 12. There was no effect of treatment or time x treatment interaction on MCH concentration.

Table 6.10.   *Effect of molybdenum or iron supplementation on mean cell haemoglobin (MCH) concentration (pg) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	9.78	9.54	9.79	0.254	NS
2	10.55	10.23	10.69	0.288	NS
4	10.84	10.73	10.76	0.225	NS
6	10.53	10.40	10.44	0.218	NS
8	10.52	10.40	10.30	0.286	NS
10	10.66	10.63	10.79	0.210	NS
12	10.88	10.91	10.98	0.161	NS

Mean Corpuscular Haemoglobin (MCH) = Haemoglobin content of the cell (pg) (Kerr, 2002)

Repeated Measures Analysis: Treatment effect   P = NS

Time effect   P = <0.001

Time X Treatment effect   P = NS

6.3.2.6. Mean Corpuscular Haemoglobin Concentration (MCHC)

There was no effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo supplementation on MCHC concentration (g / 100 ml) (Table 6.11.). Repeated measures analysis indicated that there was an effect of time (P<0.001) with all dietary treatment groups having increased MCHC from week 0 to week 12. There was no effect of treatment or time x treatment interaction on MCHC concentration.

Table 6.11. *Effect of molybdenum or iron supplementation on mean corpuscular haemoglobin concentration (g /100 ml) (MCHC) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	34.31	32.98	32.72	1.143	NS
2	36.44	34.75	35.19	1.336	NS
4	37.47	36.60	35.58	1.174	NS
6	36.37	35.41	34.55	1.123	NS
8	36.30	35.29	33.95	1.239	NS
10	36.02	35.13	34.61	1.123	NS
12	36.41	35.50	34.64	0.925	NS

MCHC (g/100 ml) = Hb (g/100 ml) / Hc (decimal fraction) (Kerr, 2002)

Repeated Measures Analysis:	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

6.3.2.7. Platelet concentration

There was no effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on platelet concentration (x10^9/l) (Table 6.12.). Repeated measures analysis indicated that there was an effect of time (P<0.05) with the control and Fe treatment groups having increased platelet concentrations from week 0 to 12. There was no effect of treatment or time x treatment interaction on platelet concentration.

Table 6.12.   *Effect of molybdenum or iron supplementation on platelet concentration (x 10^9 /l) of Scottish Blackface wethers*

| | Treatment | | | | |
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	172	128	240	57.6	NS
2	224	212	221	56.6	NS
4	245	222	245	52.4	NS
6	225	269	222	53.9	NS
8	280	273	240	46.6	NS
10	236	246	228	48.8	NS
12	208	251	211	48.5	NS

Repeated Measures Analysis: Treatment effect   P = NS

Time effect   P = <0.05

Time X Treatment effect   P = NS

6.3.2.8. White Blood cell concentration

There was no effect of 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo on WBC concentration (x 10^9 /l).

From week 8 to week 12 inclusive, there was a trend for wethers that received the 5 mg kg^{-1} Mo dietary treatment to have a lower WBC concentration compared to wethers that received the control or 500 mg kg^{-1} Fe dietary treatments, although this was not significantly different (Table 6.13.). Repeated measures analysis indicated that there was an effect of time (P<0.001) with the Fe and 5 Mo dietary treatment groups having decreasing WBC concentrations from week 0 to week 12 but there was no effect of treatment or time x treatment interaction on WBC concentration.

Table 6.13.   *Effect of molybdenum or iron supplementation on white blood cell (WBC) concentration (x 10^9 /l) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	11.45	11.03	11.50	1.355	NS
2	13.39	12.47	11.13	1.585	NS
4	12.30	10.99	10.64	1.338	NS
6	11.60	11.11	10.05	1.088	NS
8	11.49	10.21	9.66	1.051	NS
10	12.33	10.74	9.43	1.162	NS
12	11.03	9.88	8.69	0.959	NS

Repeated Measures Analysis: Treatment effect   P = NS

Time effect   P = <0.001

Time X Treatment effect   P = NS

6.3.3. Plasma copper (Pl-Cu) concentration

Wethers that received the 5 mg kg^{-1} Mo dietary treatment had higher Pl-Cu concentrations compared to wethers that received the control or 500 mg kg^{-1} Fe dietary treatments at week 2 (P<0.05), week 6 (P<0.01) and week 12 (P<0.01) respectively (Fig. 6.2.). Wethers that received the 500 mg kg^{-1} Fe dietary treatment had lower Pl-Cu concentrations compared to the wethers that received the control or 5 mg kg^{-1} Mo dietary treatments at week 10 (P<0.05) and week 12 (P<0.01). Repeated measures analysis indicated that there was an effect of time (P<0.001) with all Pl-Cu concentrations decreasing in all treatment groups from week 0. There was an effect of treatment (P<0.05) and a significant time x treatment interaction (P<0.05) on Pl-Cu concentration due to the significant decrease in Pl-Cu concentration in the Fe dietary treatment group compared to the control and 5 Mo groups.
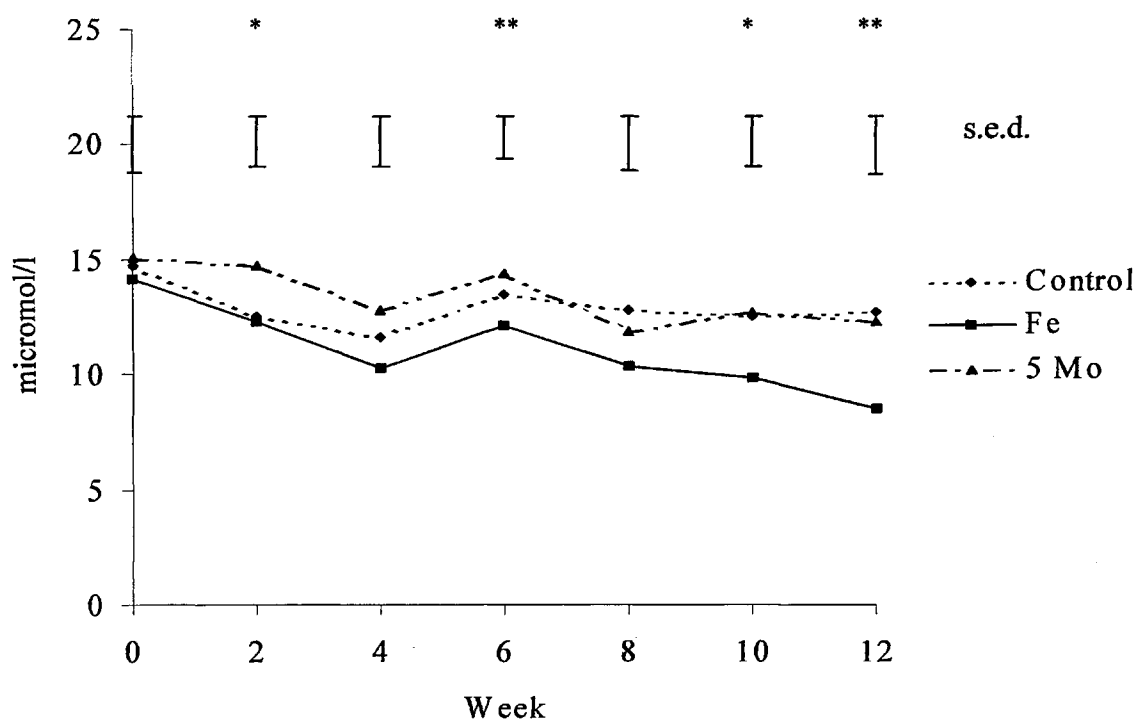
Figure 6.2 Effect of molybdenum or iron supplementation on plasma copper
concentration of Scottish Blackface wethers

Repeated measure analysis:	Treatment effect	P = <0.05
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.05

183

6.3.4. Ceruloplasmin activity

Wethers that received the control dietary treatment had higher (P<0.001) CP activity at weeks 6, 8, 10 and 12 inclusive compared to wethers that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments (Fig. 6.3). There was no difference in CP activity between the wethers that received the 500 mg kg^{-1} Fe or 5 mg kg^{-1} Mo dietary treatments at any time point. The control group had a mean CP level of 14.95 mg/dl at week 12. Repeated measures analysis indicated that there was an effect of treatment (P<0.001) with the control group having higher CP activity than the Fe or 5 Mo treatments, an effect of time (P<0.001) and a time x treatment interaction (P<0.001) on CP activity. There was a sharp decrease in CP activity from week 0 to week 12 in the Fe and 5 Mo dietary treatment groups.
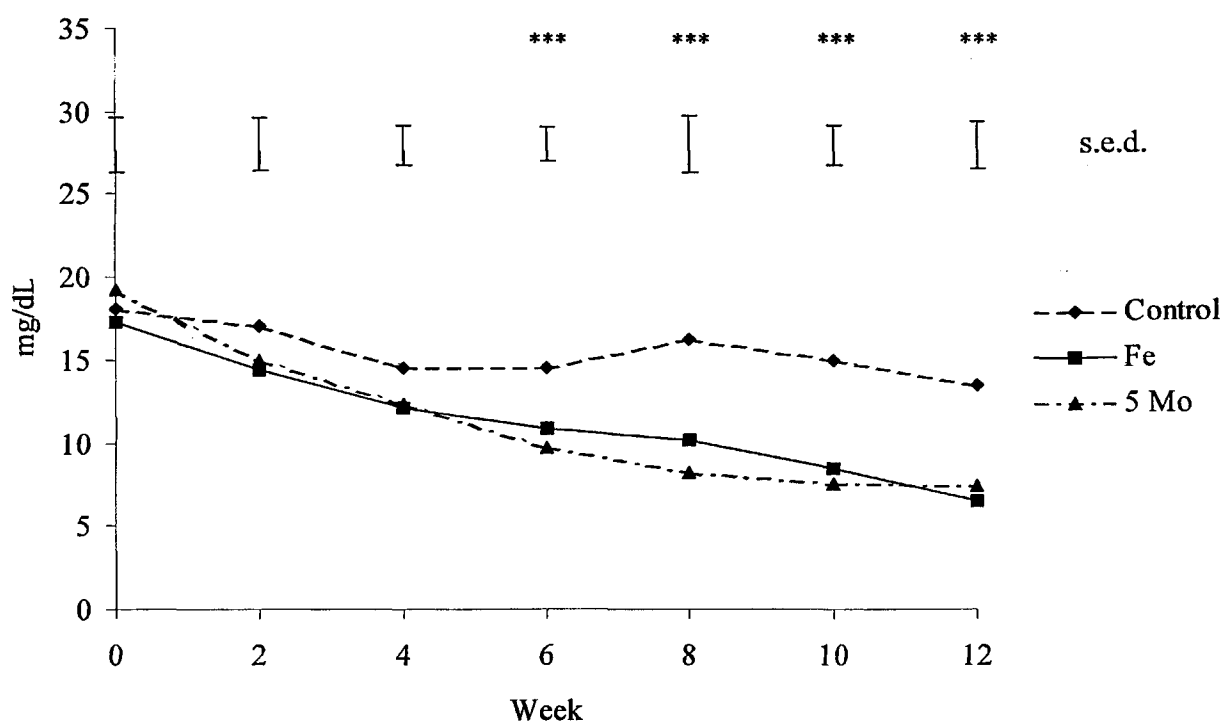
Figure 6.3. Effect of molybdenum or iron supplementation on ceruloplasmin activity of Scottish Blackface wethers

Repeated measure analysis:	Treatment effect	P = <0.001
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.001

184

6.3.5. Ceruloplasmin to Plasma Copper Ratio (CP:Pl-Cu ratio)

At week 2, wethers that received the control diet had a higher (P<0.05) CP:Pl-Cu ratio compared to the wethers that received the 5 mg kg^{-1} Mo dietary treatment (Table 6.14.). There was no difference in CP:Pl-Cu ratio between wethers that received the 500 mg kg^{-1} Fe dietary treatment and wethers that received the control or 5 mg kg^{-1} Mo dietary treatments. From week 4 to week 12 inclusive, wethers that received the control diet had a higher (P<0.001) CP:Pl-Cu ratio compared to the wethers that received the 500 mg kg^{-1} Fe or the 5 mg kg^{-1} Mo dietary treatments. Also, from week 4 to week 12 inclusive, wethers that received the 500 mg kg^{-1} Fe dietary treatment had a higher (P<0.001) CP:Pl-Cu ratio compared to wethers that received the 5 mg kg^{-1} Mo dietary treatment. Repeated measures analysis indicated an effect of treatment (P<0.001) due to the low ratios of the 5 Mo group compared to the control and Fe groups. There was an effect of time (P<0.001) and a significant time x treatment interaction (P<0.001) due to the decrease in CP:Pl-Cu ratios in all treatment groups over time, in particular, the Fe and 5 Mo groups.

Table 6.14. *Effect of molybdenum or iron supplementation on ceruloplasmin to plasma copper ratio (CP:Pl-Cu) of Scottish Blackface wethers*

	Treatment				
Week	Control	Fe	5 Mo	s.e.d.	Significance
0	1.23	1.23	1.23	0.065	NS
2	1.37^a	1.17^{ab}	1.03^b	0.106	*
4	1.26^a	1.18^b	0.97^c	0.065	***
6	1.10^a	0.89^b	0.67^c	0.050	***
8	1.29^a	0.98^b	0.69^c	0.073	***
10	1.20^a	0.85^b	0.59^c	0.058	***
12	1.13^a	0.80^b	0.59^c	0.062	***

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, ***<0.001

Repeated measure analysis:	Treatment effect	P = <0.001
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.001

6.3.6. Superoxide Dismutase (SOD) activity

There was no significant effect of 500 mg kg^{-1} Fe or the 5 mg kg^{-1} Mo on superoxide dismutase (SOD) activity at any timepoint (Fig 6.4). Repeated measures analysis indicated that there was an effect of time (P<0.001) due to the decrease in SOD activity in all treatment groups from week 0 to week 12, but no effect of dietary treatment and no time x treatment interaction on SOD activity.
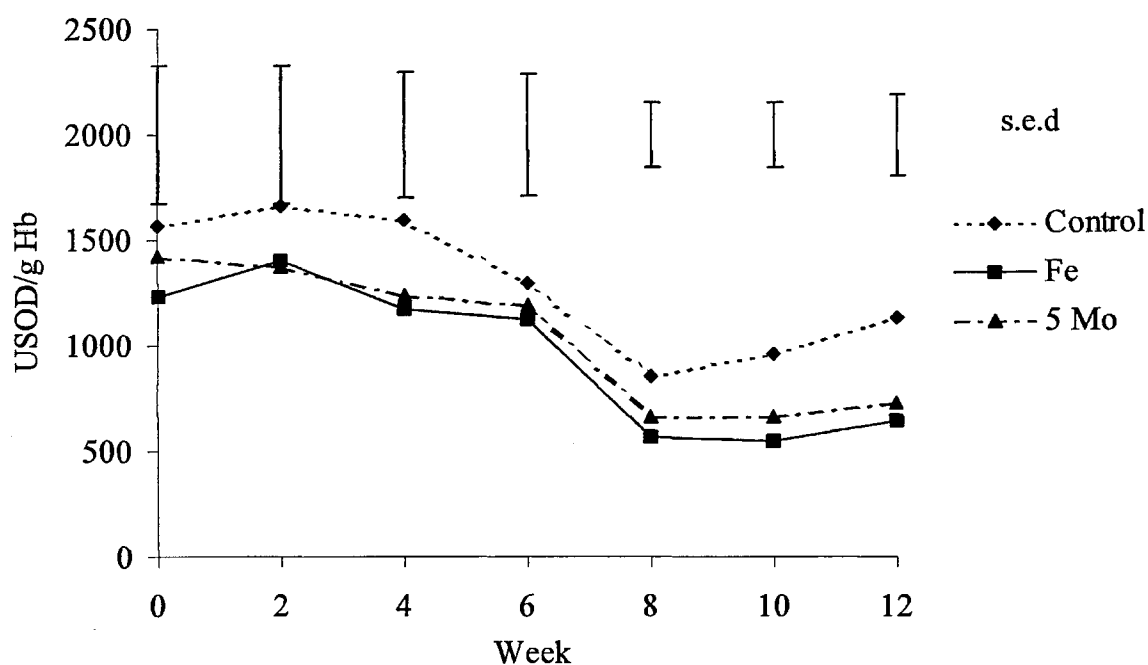
Fig. 6.4. Effect of molybdenum or iron supplementation on superoxide dismutase activity of Scottish Blackface wethers

Repeated measure analysis:	Treatment effect	P = NS
	Time effect	P = <0.001
	Time X Treatment effect	P = NS

186

6.3.7. Amine Oxidase (AMOX) activity

From week 4 to week 12 inclusive, wethers that received the control dietary treatment had higher (P<0.001) AMOX activity compared to the wethers that received the 5 mg kg^{-1} Mo dietary treatment (Fig. 6.5.). Wethers that received the 500 mg kg^{-1} Fe dietary treatment had lower (P<0.001) AMOX activity compared to wethers that received the control dietary treatment at week 2 to week 12 inclusive. At week 2, wethers that received the 500 mg kg^{-1} Fe dietary treatment had lower (P<0.001) AMOX activity compared to the wethers that received the 5 mg kg^{-1} Mo dietary treatment. Repeated measures analysis indicated an effect of treatment (P<0.001) due to the decrease in AMOX activity in the Fe and 5 Mo treatment groups compared to the control group and an effect of time (P<0.001) and a time x treatment interaction (P<0.001).
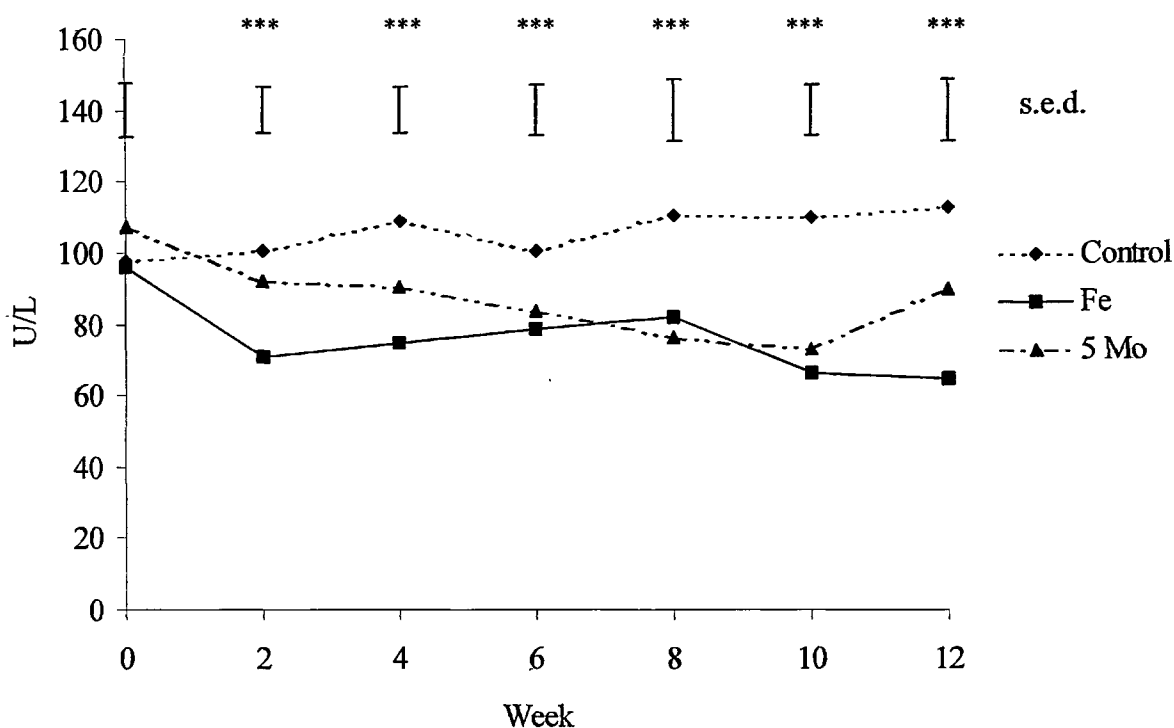
Figure 6.5.   Effect of molybdenum or iron supplementation on amine
oxidase activity of Scottish Blackface wethers

Repeated measure analysis:	Treatment effect	P = <0.001
	Time effect	P = <0.001
	Time X Treatment effect	P = <0.001

6.3.8. Trace element content of the liver

Wethers that received the Fe dietary treatment had lower (P<0.05) liver Cu concentration than the wethers that received the control dietary treatment (Table 6.15). There was no difference in liver Cu concentration for lambs that received the control dietary treatment compared to the wethers that received the 5 mg kg^{-1} Mo dietary treatment. There were no treatment differences in liver Fe, Zn or Mn concentrations. Lambs that received the 5 mg kg^{-1} Mo dietary treatment had higher (P<0.001) liver Mo concentrations compared to the wethers that received the control or 500 mg kg^{-1} Fe dietary treatments. There were no differences in liver Mo concentrations between the control or 500 mg kg^{-1} Fe dietary treatment groups.

Table 6.15. *Effect of molybdenum or iron supplementation on trace element content in the liver* (μg/g DM ± s.e.d.) *of Scottish Blackface wethers*

	Treatment				
	Control	Fe	5 Mo	s.e.d.	Significance
Element					
Cu	146.89[b]	51.87[a]	107.10[ab]	32.401	*
Fe	519.60	576.97	487.01	64.807	NS
Mo	4.66[a]	4.41[a]	7.22[b]	0.368	***
Zn	167.95	149.91	159.81	9.050	NS

[a,b] Means within a row with different superscripts are significantly different (P<0.05)
* <0.05, **<0.01, ***<0.001

6.3.9. mRNA expression in the liver

Optimum primers and conditions were determined to be CerF1/R1 and baF1/R1 with an anneal of 60°C. Amplification of DNA and RNA extracted from sheep livers produced the expected size PCR products when amplified with CerF1/R1 and baF1/R1 either alone or in combination. Primer pair CerF1/R1 failed to amplify DNA. These primers were designed to amplify across Intron 1 that were of unknown size and may have been too large to allow amplification of a DNA product under these conditions (Fig. 6.6.).
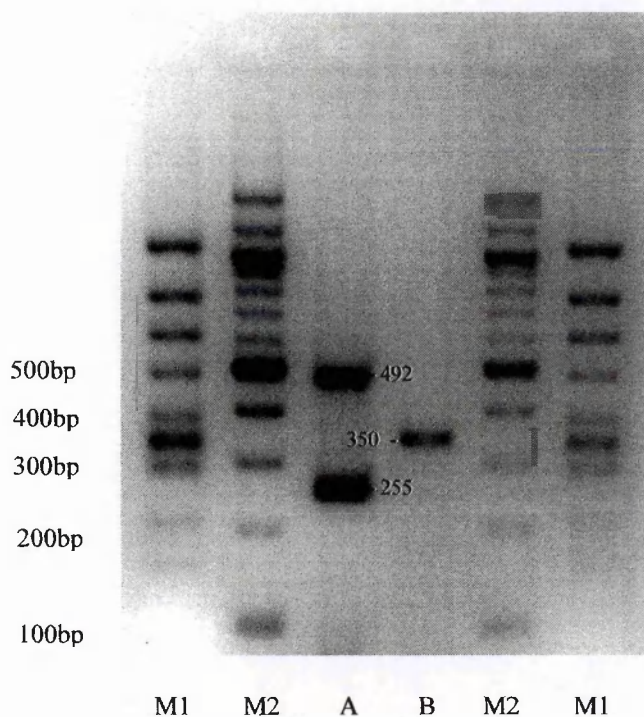
Fig. 6.6.

Agarose gel electrophoresis showing products from RT-PCR analysis of RNA and DNA amplification from liver extractions of Scottish Blackface wethers using primer pairs CPF1/R1 and baF1/R1.

M1 ØX 174 *Hinc* II ((New England Biolabs, Hitchin, UK)
M2 100bp ladder
A RNA (CP, 492bp ; ba, 255bp)
B DNA (ba, 350bp)

RT-PCR amplification of mRNA from all wether replicates followed by ANOVA on the CP/ba ratio indicated that there was no effect of dietary treatment on mRNA expression in the liver (Table 6.16 and Fig. 6.7.). No DNA products were detectable after gel electrophoresis indicating that there was no DNA contamination of the RNA extractions (Fig. 6.7.)

189

Table 6.16.   Effect of molybdenum or iron supplementation on mRNA Cer/ba ratio

Treatment

Control	Fe	5 Mo	s.e.d.	Significance
0.930	0.932	0.957	0.0854	NS
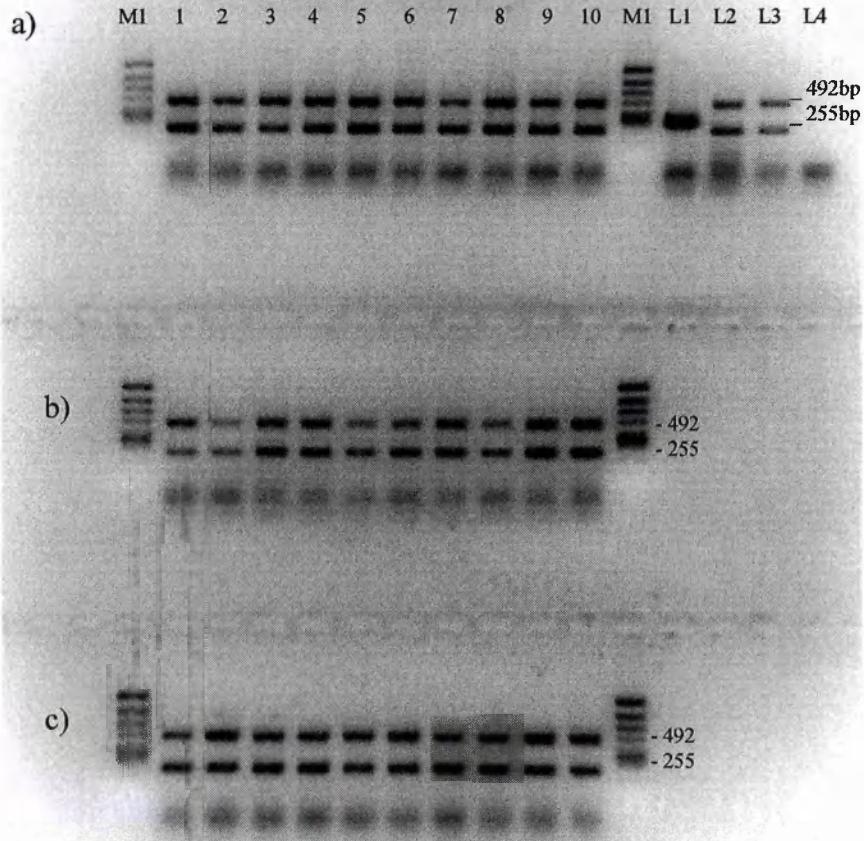
Fig. 6.7. Agarose gel electrophoresis showing products from RT-PCR analysis of RNA liver extractions from Scottish Blackface wethers amplified by primers specific to ceruloplasmin (CP) and β-actin (ba) genes: (a) control diet (b) 500 mg kg^{-1} iron diet (c) 5 mg kg^{-1} Mo diet

M1	ØX 174 *Hinc* II (New England Biolabs, Hitchin, UK)
1-10	Individual liver replicates
L1	RT-PCR Kit (+) control (323bp)
L2	Wether 706 - Pure extraction known to amplify
L3	Wether 706 - 10^{-1} extraction known to amplify
L4	RT-PCR Kit (-) control

492bp	CPF1/R1
255bp	baF1/R1

6.4. Discussion

Chemical analysis of the control diet was within the normal range for ruminant feedstuffs as specified by the AFRC (1993). The basal diet contained 5.5 mg kg^{-1} Cu and all other mineral elements within the basal diet were considered to be adequate for adult male sheep (AFRC, 1980), except for selenium levels which were higher than normal requirements.

Similar to results from chapters 3 to 5 inclusive and work by Phillippo *et al.* (1987a) in calves, dietary Mo or Fe fed to sheep over a twelve week period had no effect on growth rate or performance, induced no clinical symptoms and had no significant effects on the haematology profile in Scottish Blackface wethers. However, dietary Mo, and to a lesser extent dietary Fe, did alter the Cu status of the sheep used in this study. In accordance with chapters 3 and 5 and work by Du Plessis *et al.* (1999a), it was found that dietary Mo significantly increased Pl-Cu concentrations with a subsequent decrease in CP activity. Dietary Fe was found to reduce CP activity to a similar degree as dietary Mo but the mechanism by which this occurs is unclear. This study showed that dietary Fe reduced both liver Cu and Pl-Cu concentrations although dietary Mo decreased liver concentrations but increased Pl-Cu concentrations. Therefore, the mechanism by which dietary Fe alters Cu metabolism remains unclear. The presence of TM within the blood was not confirmed experimentally using the TCA-insoluble Cu method due to the unreliability of this test (Van Niekerk and Van Niekerk, 1989a). The CP:Pl-Cu ratio as proposed by Mackenzie *et al.* (1997) was used as an additional indicator of Cu status and was found to be very useful in identifying animals that had severe TM problems as a consequence of being fed dietary Mo. Similarly, the CP:Pl-Cu ratio indicated that wethers fed dietary Fe also had very low ratios but the reason for this remains unclear. Serum amine oxidase activity remained higher in control fed lambs but SOD activity remained unaltered by dietary treatment, similar to findings from chapters 3 and 5.

As discussed, the mechanism by which dietary Mo alters CP activity remains unclear, and therefore, this study aimed to establish if dietary Mo affected mRNA CP expression directly. Although, expression of the CP protein is known to occur at a constitutive rate in hepatocytes (Gitlin *et al.*, 1992), CP mRNA expression has also been found to a lesser extent in the spleen (Kalmovarin *et al.*, 1991), mammary gland (Wooten *et al.*, 1996) uterus (Thomas *et al.*, 1995), lung (Fleming *et al.*, 1991) and testis (Aldred *et al.*, 1987).

β-actin was used in this study as a housekeeping gene to quantify relative expression of the CP gene. β-actin has been used previously by other authors as an internal standard to monitor the relative expression of genes (Serazin-Leroy *et al.*, 1998) and therefore, primers were designed across a known intron from previous mRNA sequences from sheep and rat and a DNA sequence from a pig. CP primers suitable for RT-PCR were designed from conserved regions within mRNA from sheep, mouse and rat, and also rat DNA exon 1. Unfortunately, limited DNA sequences were available from different species to determine intron positions and sizes. Therefore, the β-actin primers were designed from a DNA sequence from pig that allowed prediction of a DNA PCR product size. For CP, DNA from the rat (Exon 1) was used which indicated where the first intron occurred, but not its size. The primers were designed across known intron sites so that size difference between generic DNA and complimentary DNA could be identified. Using liver samples, β-actin produced PCR products of the expected size using CerF1/R1 and baF1/R1. For CP, RNA also produced the expected size products of RNA, but no DNA products. It is not known why this failure to produce a DNA product occured but it may have been due to the presence of one or more large introns which resulted in a CP DNA product too large to be amplified under the conditions used in the RT-PCR. There was no evidence of DNA contamination of the RNA extractions as identified by the lack of a 350bp product from β-actin DNA.

Following RT-PCR amplification in this study, results indicated that there was no significant difference in relative mRNA expression of CP in the livers of Scottish Blackface wethers when assessed using a CP/ba ratio. No differences occurred in expression of CP, suggesting that modifications in CP activity, as often seen in ruminant animals consuming high intakes of Mo or Fe, either occur as a post-transcriptional defect or due to some other form of inhibition post secretion (Bissig *et al.*, 2001). Therefore, the mRNA expression of CP did not account for the differences observed in the reduction in serum CP activity. The results from this study confirm those of Gitlin *et al.* (1992) who found that the Cu status of rats was independent of the basal level of hepatic CP mRNA expression. Copper-deficient and Cu adequate rats were found to secrete newly synthesised CP at identical rates, despite little or no holo-CP in the hepatocytes. Other studies with Cu-deficient and Cu-adequate rats found that the rate of biosynthesis and secretion of apo-CP release occurred at the same rate, regardless of Cu availability (Holtzman and Gaumnitz, 1970). If Cu is not available during biosynthesis of CP, an unstable apo-protein, which lacks oxidase activity is produced and secreted in the plasma (Gitlin *et al.*, 1992). Similar results by Nakamura *et al.* (1995) indicated that CP protein biosynthesis was independent of intracellular Cu content *in vivo* using cultured hepatocytes from Long-Evans Cinnamon (LEC) rats. A method of identifying apo and holo-CP was described by Sato and Gitlin (1991), although the proportion of apo and holo-protein were not assessed in this study. CP activity measured in this study relates to active holo-protein.

Another possible mechanism to account for the decrease in CP activity observed in this study may have a failure to incorporate Cu into the newly synthesised apo-protein to produce the holo-protein as described in LEC rats (Sato *et al.*, 1993). The secreted apo-protein may be deficient in Cu and therefore lack true oxidase activity, which may account for the reduction in CP activity as observed in this study. Genetic defects in the ATPase ATP7B (Bull *et al.* 1993), account for the reduction in Cu incorporation into the apo-CP

193

protein of human patients with Wilson's Disease. Wilson's Disease is an autosomal recessive Cu toxicity disorder affecting the liver and nervous system (Sternlieb, 1993). Due to mutations in the protein, Cu cannot be incorporated into CP, resulting in low or absent serum CP due to reduced Cu incorporation into CP in the hepatocyte (Gibbs and Walshe, 1979) and an accumulation of Cu in the liver (Schaefer and Gitlin, 1999). The role of ATP7B is to incorporate Cu into CP and to regulate Cu levels within the hepatocyte to prevent Cu toxicity by the direct removal of Cu into the bile (Mercer, 2001). Studies by Lockhart and Mercer (1999) found that the function of ATP7B (to transport Cu into the secretory pathway for incorporation into CP) occurred in a similar way for sheep as that of human ATP7B. Certain breeds of sheep display an unusual pattern of Cu metabolism (Lockhart and Mercer, 1999). Genetic variations in susceptibility to Cu toxicosis in sheep breeds has been found to not be due to a gross malfunction of ATP7B (Lockhart *et al.*, 2000a), but more likely as a defect in Cu transport or trafficking within the hepatocyte. The copper chaperone Sheep Atx1 Homologue *(SAH)* was found to interact with ATP7B, facilitating in transporting Cu in the secretory pathway of the hepatocyte (Lockhart *et al.* 2000a). Two forms of the sheep Wilson disease (ATP7B) homologue (sWNDa and sWNDb) were identified by Lockhart *et al.* (2000b) and provided a possible explanation for differences in biliary Cu excretion and retention of Cu in the liver by different breeds of sheep prone to Cu toxicosis. However, there is no evidence within the literature of differential expression of ATP7B between breeds. These two isoforms may account for the normal plasma CP levels observed in sheep prone to Cu toxicosis (such as North Ronaldsay and Texel). The results also suggested that the tendency of sheep to accumulate Cu in the liver was not due to a gross alteration in the structure or expression of ATP7B. Therefore, the expression of ATOX1 and SAH require further investigation regarding their role in facilitating Cu transport within hepatocytes.

The gram positive bacteria *Enterococcus Hirae* has been found to encode two different ATPases; CopA and Cop B (Bissig *et al.,* 2001). Cop A is involved in the uptake of Cu under Cu limiting conditions and CopB is involved in the removal of Cu as it approaches toxic levels within the cell, similar to ATP7B. Bissig *et al.* (2001) found that the CopB ATPase was strongly inhibited by di, tri and tetrathiomolybdate but this inhibition could be reversed by Cu administration. Work by Ogra *et al.* (1999) also found that tetrathiomolybdate adminstration to Long Evans Cinammon (LEC) rats was effective at reducing Cu accumulation. However, the reduced amounts of Cu in CP were believed to be due to the sequestering of Cu from the Cu chaperones, rather than directly removing Cu from the CP enzyme. Therefore, more research also needs to be undertaken to determine how TM may reduce the Cu available to the specific Cu chaperones or if TM directly affects the expression of ATP7B within the hepatocyte.

Dietary Fe was found to significantly reduce liver Cu concentrations in this study when compared to the control fed wethers. Similar to findings of chapters 3 to 5 inclusive, dietary Fe also acts as a Cu antagonist in reducing liver Cu stores. Dietary Mo reduced liver Cu concentrations, but to a lesser extent than dietary Fe, confirming that the antagonistic effect of dietary Fe on Cu metabolism requires further investigation. Dietary Mo was found to significantly increase the Mo content in the liver compared to the control or Fe dietary treatment groups, but similar to chapter 5, the consequence of Mo accumulation in the liver remains unclear.

6.5. Conclusion

In conclusion, the results from this study confirmed those of chapters 3 to 5 inclusive that dietary Mo or Fe altered Cu status of Scottish Blackface wethers but induced no clinical symptoms. The mRNA expression of CP was found to be produced at a constitutive rate in the liver in accordance with findings of Gitlin *et al.* (1992) and was not affected by dietary Mo or Fe. Therefore, further studies are required to determine how dietary Mo and Fe may reduce CP activity both within the hepatocyte, post secretion or due to the systemic effect of TM within the blood or tissues.

Chapter 7

General Discussion

7.1. Introduction

These series of studies were designed to investigate the effects of molybdenum (Mo) and iron (Fe) on Cu metabolism and physiology of sheep. Although this thesis clearly demonstrates that Mo, and to a lesser extent, Fe, are antagonistic minerals in relation to Cu metabolism in ruminant animals, the subsequent consequences of these mineral interactions in the blood and tissues still remain unclear. Early studies by Ferguson *et al.* (1943) reported high Mo contents in the 'teart' pastures of Somerset was the likely cause of severe scouring, changes in coat colour and texture, and general loss of condition in cattle, and to a lesser degree in sheep. Blakemore and Venn (1950) described a 'conditioned' Cu deficiency on a farm in Suffolk where adult cattle were grazed on poor quality, sandy soil pastures but which contained seemingly adequate Cu levels. Similar to Ferguson *et al.* (1943), Blakemore and Venn (1950) described clinical symptoms resembling those of cattle grazing pastures of high Mo contents including intermittent diarrhoea, poor fertility, loss of condition and emaciation in adults and small calves at birth. The link between dietary Mo and clinical Cu deficiency during 1950's had not been confirmed. Later reports by Munro (1957) described infertility problems in dairy herds between 1952 and 1955 in cattle grazing a range of light sandy to heavy loams soils. Again, clinical symptoms including changes in coat texture and colour and general unthriftiness in cattle were observed, although were not directly attributed to high dietary Mo intakes. In all of these early studies, all herds responded positively to Cu supplementation, either by improvements in reproductive performance or in the general condition of the animals. The relationship between clinical Cu deficiency and fertility in cattle has now been firmly established (Phillippo *et al.,* 1982, 1987b; Van Niekerk and Van

Niekerk, 1989b) although the reason by which Mo and Fe exert such different clinical effects with similar Cu status is not known.

A potential mechanism by which dietary Mo may reduce Cu absorption was described by Suttle (1974a) and Dick *et al.* (1975) who proposed that dietary Mo interacted with Cu and sulphur (S) within the rumen to form a series of di-, tri- and tetra-thiomolybdate (TM) complexes. These TM complexes had a high affinity for Cu, and consequently, reduced Cu absorption within the digestive tract with Cu subsequently being excreted in the faeces. When TM was present in excess, it could be absorbed into the blood and tissues as their respective ammonium salts. Work at the Rowett Institute by Humphries *et al.* (1983), Bremner *et al.* (1983; 1987) and Phillippo *et al.* (1987a; 1987b) proposed that clinical Cu deficiency was not due to a low Cu status as suggested by Suttle (1974a), but was primarily due to the formation and subsequent absorption of TM produced from high dietary Mo intakes, with the net effect of systemically altering Cu-dependent enzyme activities and directly altering reproductive performance. Therefore, the four experiments undertaken within this study were designed to further investigate the antagonistic effects of dietary Mo and Fe and the subsequent systemic effects of absorbed TM, using sheep as a model.

7.2. Composition of the basal diets

In order to be able to discuss the observed differences due to dietary Mo or Fe in this series of studies, it is appropriate to compare the basal diets of the four experiments described in chapters 3 to 6 inclusive. Composition of the basal diets is presented in Table 7.1.

Table 7.1   Mineral composition of the basal diets (DM) - Chapters 3 to 6 inclusive

	Chapter 3	Chapter 4	Chapter 5	Chapter 6
Calcium (g kg^{-1})	8.60	10.60	8.30	9.90
Phosphorus (g kg^{-1})	3.00	4.50	3.80	4.10
Sulphur (g kg^{-1})	2.20	3.00	2.70	2.80
Magnesium (g kg^{-1})	1.60	2.00	1.70	3.30
Copper (mg kg^{-1})	5.47	8.59	6.09	5.50
Molybdenum (mg kg^{-1})	1.29	0.88	0.94	0.67
Iron (mg kg^{-1})	167.35	197.18	194.22	206.69
Zinc (mg kg^{-1})	97.44	67.90	59.99	57.44
Cobalt (mg kg^{-1})	0.52	1.65	2.19	1.41
Selenium (mg kg^{-1})	0.0065	0.076	0.09	0.40
Manganese (mg kg^{-1})	98.12	86.51	59.71	72.19

Mineral composition of the basal diets presented in Table 7 indicates that the calcium, phosphorus and magnesium contents of the basal diets in chapters 3 and 6 were similar to the recommendations for sheep as specified in ARC (1980). The sulphur content of the basal diets was approximately 2 to 3 g kg^{-1} DM as formulated for during diet composition. Suttle (1974) found that dietary S intakes above 2 g kg^{-1} reduced availability of Cu in the diet. The zinc, cobalt and manganese contents of all basal diets were all within recommended ranges for sheep (ARC, 1980). The Cu content of the basal diet in chapter 4 was higher than the formulated 5 mg kg^{-1} Cu. Therefore, this may account for the lack of

effect on immune function as described in chapter 4 and will be discussed in section 7.4. The high Cu:Mo ratio in chapter 4 may have reduced the antagonistic interaction of dietary Mo and the subsequent formation and absorption of TM. Molybdenum contents of all basal diets were within recommended ranges for sheep (ARC, 1980). However, it is not possible to predict from these studies whether these levels of Mo may alter Cu metabolism as part of a TM complex, or if they interact to exacerbate the effect of Fe on Cu metabolism. Selenium contents of the basal diets in chapters 3 to 5 inclusive were marginally deficient and in chapter 6, above recommended levels as specified by ARC (1980). Iron contents of all basal diets were relatively similar and would have not contributed independently to the alterations in Cu status observed in the Fe supplemented sheep in chapters 3 to 6.

Variations in mineral composition within the basal diets in chapters 3 to 6 may be attributable to the dietary components used in the formulation of the diets. These differences were due to availability of the feedstuff and time of year in which the study was undertaken.

7.3. Determination of Cu status

Accurately diagnosing the true Cu status of a ruminant animal has been found to be problematic due to the lack of correlation between clinical symptoms and levels of Cu within blood, plasma and tissues. Therefore, all studies described within this study assessed the Cu status of all animals using a variety of methods, and to establish how these parameters were affected by dietary Mo or Fe.

Plasma Cu concentration (Pl-Cu) is currently the most commonly used diagnostic indicator of Cu status of an animal, however, Pl-Cu concentration alone has been deemed not to accurately reflect Cu status (Herdt *et al.*, 2000). It has traditionally been accepted that a low Pl-Cu concentration (<8 μmol l^{-1}) would indicate a Cu deficiency, although Pl-Cu concentrations between 12-24 μmol l^{-1} are currently considered to be within a 'normal' reference range for sheep. According to Suttle (1974), Mo reduced Cu uptake in the small intestines. In this series of trials, dietary Mo has been found to increase Pl-Cu concentrations, not decrease it. Therefore, Pl-Cu concentration did not reflect this dietary antagonism. Terada *et al.* (1995) demonstrated that 90-95% of Pl-Cu is in CP with Gitlin *et al.* (1992) reporting that CP is a constitutively produced protein in rats, and as reported in chapter 6, in sheep. The increase in Pl-Cu concentration in Mo supplemented sheep cannot be explained by an increase in liver Cu release alone as the main release mechanism by the liver into blood is via CP. This has also been verified in humans suffering from Wilson's Disease where they have the inability to incorporate Cu into CP due to a defect in the ATPase, ATP7B.

Pl-Cu concentrations were found to increase due to the presence of dietary Mo in chapters 3 and 5, confirming findings of Bremner and Young (1978), Van Niekerk and Van Niekerk (1989a) and Du Plessis *et al.* (1999). Dick *et al.* (1956) suggested that an increase in Pl-Cu was often coupled with a decrease in liver Cu concentration. In chapters 3 to 6 inclusive, it was found that sheep that received the control diets maintained liver Cu concentrations considered to be within normal reference ranges (100-500 μg g^{-1} DM) by the end of the

experimental periods. In contrast, it was found that in chapters 3 to 6 inclusive, dietary Mo significantly reduced liver Cu concentrations to such an extent that by the end of each experimental period, lambs in all three experimental studies had liver Cu concentrations indicative of a Cu deficiency using current reference ranges (less than 100 mg kg^{-1} DM). Although these increases in Pl-Cu concentration and the decreases in liver Cu concentration were observed in these studies due to dietary Mo, no clinical symptoms were evident in any of the sheep in chapters 3 to 6. Wentink *et al.* (1999) suggested that relying only on blood Cu concentrations alone may lead to false diagnosis of the Cu status of a ruminant animal. Therefore, Pl-Cu concentrations alone should be viewed with caution as the Cu bound to TM may not be 'available' within the plasma (Price *et al.*, 1987), but may ultimately produce diagnostic readings considered to be within the 'normal' reference ranges for ruminant animals. The lack of effect of dietary treatment on Pl-Cu concentration in chapter 5 may have been due to the high Cu concentration in the basal diet, reducing the antagonistic effects of the dietary Mo and increasing the Cu:Mo ratio.

Therefore, observations in chapter 3 and 5 contradict those of Humphries *et al.* (1983) who found that dietary Mo decreased plasma and liver Cu concentrations in association with alterations in clinical symptoms. These changes included alterations in hair texture and colour and reduced growth rates compared to Fe supplemented or control heifers. Humphries *et al.* (1983) suggested that clinical symptoms were not due to a low Cu status but were as a result of high dietary Mo intakes that encouraged the formation of TM. Later work by Phillippo *et al.* (1987b) found that although Cu status was reduced in Mo, Fe and Mo x Fe supplemented heifers, only Mo supplemented animals had observed systemic effects resulting in changes in delayed puberty, reduced conception rate and reductions in hormone release.

Although dietary Fe did reduce liver Cu concentrations in chapters 3 to 6 inclusive, these reductions were not as severe as in sheep that received dietary Mo. No increases in Pl-Cu concentrations were observed in the Fe dietary treatment groups as observed in sheep

receiving dietary Mo. There was a trend for Pl-Cu concentrations to be reduced in sheep receiving the Fe dietary treatment, suggesting an alternative mechanism by which dietary Fe affects Cu metabolism to that of dietary Mo. Suttle *et al.* (1984) suggested that Fe interacted with sulphide in the rumen restricting Cu absorption from the small intestine. Work by Gooneratne *et al.* (1994) confirmed that dietary Mo increased biliary Cu excretion in cattle. Studies by Gooneratne and Christensen (1984) and Ke and Symonds (1987) found that intravenous administration of ammonium TTM increased biliary Cu excretion in sheep. Therefore, the current series of studies indicates that dietary Fe not only reduces Cu absorption but also reduces CP activity. Dietary Mo is also absorbed, possibly as TM and also acts systemically in the blood and tissues.

Ceruloplasmin (CP) is also used diagnostically as an indicator of Cu status (normal range 12-24 mg/dl) due to its' relative sensitivity to dietary antagonists (Mackenzie *et al.*, 1997). These series of studies found that in chapters 3, 4 and 6, mean CP activity of the control sheep was higher than the respective animals that received dietary Mo or Fe, in accordance with work by Humphries *et al.* (1983). It was reported by Mason *et al.* (1982) that duodenal infusions of Mo produced rapid decreases in plasma CP activity in sheep. Under normal conditions, Cu is transported in the blood plasma primarily bound to CP (90-95%) (Terada *et al.*, 1995) and to a lesser extent, bound to albumin, metallothionein and other amino acids. Mason *et al.* (1986; 1988) reported that in ruminant animals exposed to dietary Mo, a new Cu fraction associated with albumin appeared. This was believed to be due to albumin bound TM acting as powerful ligands by removing Cu from Cu proteins including metallothionein within the liver. Early studies by Kelleher *et al.* (1983) and Hynes *et al.* (1984) found that TM bound preferentially to albumin rather than CP. Later studies by Lannon and Mason (1986) found that tri or TTM administration to steers produced decreases in CP activity coupled with an increase in Cu bound to plasma albumin. Gooneratne *et al.* (1981b) suggested that after prolonged exposure to TM, CP

203

concentrations decrease in the plasma. Work by Komatsu *et al.* (2000) confirmed the finding that circulating Cu-TTM complexes are selectively bound to albumin, suggesting an alteration in the fractionation of Cu in the blood. Work by Ogra *et al.* (1999; 2000) found that Cu within CP was decreased by TM in Long Evans Cinnamon (LEC) rats and the Cu found in the plasma was in the form of a Cu-thiomolybdate-albumin complex. CP was also found to be reduced by TM which was suggested to be as a result of TM sequestering Cu from its' respective Cu chaperone, ATOX1, within the liver and not as a result of direct removal of Cu from the CP enzyme (Ogra *et al.*, 1999; 2000). Again, this finding confirms early studies of Kelleher and Mason (1986) that CP is reversibly inhibited by TM. Evidence from this study and the work cited above clearly show that dietary Mo, in the form of TM, may inhibit CP activity by a number of possible mechanisms:

1. Direct inhibition of CP that exists in plasma

2. Removal of Cu from CP present in plasma

3. Reduction in Cu available to the trans-golgi network (TGN) for incorporation into CP

Possibly, TM works by acting at all three levels. However, what can be concluded from chapter 6 is that dietary Mo or Fe does not alter the expression of the CP gene. Previous studies have found that sheep with low CP activities due to dietary Mo responded well to Cu supplementation (Moeini, 1997).

The most sensitive indicator of the effects of dietary Mo used in this series of experiments was the CP:Pl-Cu ratio as described by Mackenzie *et al.* (1997) for cattle and sheep which took in to consideration the systemic effect of absorbed TM. A CP:Pl-Cu ratio of <2.0 was suggested by Mackenzie *et al.* (1997) to be indicative of free TM being absorbed into the blood, reducing the activities of the Cu enzymes. Based on the predicted CP:Pl-Cu ratio of 2:1, results from chapters 3 to 6 found significant differences between dietary treatments in the CP:Pl-Cu ratio and identified those animals that received dietary Mo to have been

possibly affected by TM, although TM was not directly measured. Mackenzie *et al.* (1997) suggested that CP:Pl-Cu ratios of <1.5 were indicative of TM problems, with ratios of <1 indicating severe TM problems. Results obtained in chapters 3 to 6 inclusive indicated that all sheep that received dietary Mo had CP:Pl-Cu ratios by the end of the respective experimental periods to be indicative of TM problems which were not apparent from either the Pl-Cu or CP concentrations alone. Although CP:Pl-Cu ratios in sheep that received dietary Fe were found to decrease, this may have been due to an alteration in Cu metabolism resulting in a decrease in available Cu due to Cu bound within a Cu x Fe complex. Therefore, the use of the CP:Pl-Cu ratios may be more useful as a diagnostic aid than other current methods in identifying animals with systemic alterations in their blood due to high dietary Mo or Fe.

Using data obtained from chapters 3 to 6 inclusive, Fig 7.1 indicates the relationship between the CP:Pl-Cu ratio and liver Cu concentrations for all treatment groups at the end of each respective trial. These results indicate that the regression coefficient of the CP:Pl-Cu ratio to liver Cu concentration was poorly correlated ($r^2 = 0.2444$; y=103.72x) although Fig. 7.1 indicates that the CP:Pl-Cu ratios from the control treatment groups were often associated with higher liver Cu concentrations. It is clear that both the dietary antagonists Mo and Fe reduced liver Cu concentrations when compared to the respective control groups but a reduction in the CP:Pl-Cu concentration was not always apparent. Figure 7.1 also indicates that by the end of each experimental period, the CP:Pl-Cu ratios for all dietary treatment groups (albeit one control subject) were within the 0.5 to 1.5 range. This data suggests that the ratio proposed by Mackenzie *et al.* (1997) for cattle may need adjusting for sheep as this data indicates that control animals that were not subjected to the dietary antagonists Mo or Fe had ratios lower than the 2.0 as proposed by Mackenzie *et al.* (1997).

Fig. 7.1. *The relationship between the CP:Pl-Cu ratio and liver Cu concentrations from sheep that received diets containing dietary Mo or Fe (using data acquired from chapters 3 to 6 inclusive).*
(Key: Control treatment - black circle; Fe treatment – blue diamond; 2 Mo – green square; 5 Mo – red circle ; 10 Mo – grey diamond)

Cu-Zn SOD (SOD_1) reference ranges in sheep range from 1100-3000 U g/Hb (D.V. Illingworth, personal communication) based on studies by Suttle and McMurray (1983) and Suttle (1986). SOD activity has previously been suggested to be more reliable indicator of Cu status than other diagnostic procedures due to the slow rate of decline during a Cu deficiency (Suttle and McMurray, 1983). The antagonistic effect of TM in reducing Cu enzyme activities would suggest that SOD activity should decline at a similar rate to that of CP. However, it must be taken into consideration that CP is free within the blood plasma whereas SOD is contained within the erythrocyte. TM is known to preferentially complex to free Cu ions, but little is known regarding the mechanism by which TM may cross cell membranes and sequester the Cu from SOD within the erythrocyte. Ogra *et al.* (1996) found that *in vitro* studies using liver supernatants from

Fig. 7.1.   *The relationship between the CP:Pl-Cu ratio and liver Cu concentrations*
from sheep that received diets containing dietary Mo or Fe (using data
acquired from chapters 3 to 6 inclusive).
(Key: Control treatment - black circle; Fe treatment – blue diamond; 2 Mo
– green square; 5 Mo – red circle ; 10 Mo – grey diamond)

Cu-Zn SOD (SOD_1) reference ranges in sheep range from 1100-3000 U g/Hb (D.V.

Illingworth, personal communication) based on studies by Suttle and McMurray (1983)

and Suttle (1986). SOD activity has previously been suggested to be more reliable

indicator of Cu status than other diagnostic procedures due to the slow rate of decline

during a Cu deficiency (Suttle and McMurray, 1983). The antagonistic effect of TM in

reducing Cu enzyme activities would suggest that SOD activity should decline at a similar

rate to that of CP. However, it must be taken into consideration that CP is free within the

blood plasma whereas SOD is contained within the erythrocyte. TM is known to

preferentially complex to free Cu ions, but little is known regarding the mechanism by

which TM may cross cell membranes and sequester the Cu from SOD within the

erythrocyte. Ogra *et al.* (1996) found that *in vitro* studies using liver supernatants from

LEC rats indicated that TTM did not remove Cu from SOD as long as Cu bound to metallothionein was present. Results from chapter 3, 4 and 6 indicated that SOD activity was relatively insensitive to either dietary Mo or Fe and few reductions were observed. The lack of effect on SOD activity may also have been due to the experimental time period of these four studies. The life span of erythrocytes in lambs is between 90-120 days (Kerr, 2002) and therefore, the effect of dietary Mo on erythrocyte populations and reductions in SOD activity may only become apparent after longer exposure to these dietary antagonists. Bremner *et al.* (1987) found similar results to those observed in chapters 3, 4 and 6, in that SOD activity was unaffected by dietary Mo or Fe treatments in weaned calves. Rigo *et al.* (1978) suggested that Cu was incorporated into SOD during erythropoeisis. Reductions in Cu availability due to Cu being bound to TM within the plasma may therefore reduce the Cu available for incorporation into SOD during erythropoesis. This may further explain why SOD activities are often observed to decline after long periods of time. Moeini (1997) found that dairy heifers supplemented with Mo and Fe had significantly lower SOD activities compared to the respective control heifers after 48 days. However, this was due to the SOD activity in the control heifers increasing after 48 days, with no subsequent change in SOD activity of the treated heifers from day 0. Humphries *et al.* (1983) found that erythrocyte SOD activities of heifer calves declined by approximately 50% after 16 weeks of being fed diets containing dietary Mo or Fe, with further decreases occurring between 16-32 weeks. Similarly, Andrewartha and Caple (1980) found that lambs that received dietary Mo had 40% lower SOD activities after 90 days compared to control sheep. In addition, erythrocyte SOD activity was slow to respond after discontinuation of the Mo treatment diet, confirming the slow response to changes in SOD activity. Both Andrewartha and Caple (1980) and Phillippo *et al.* (1982) found that erythrocyte SOD activities responded to Cu supplementation. However, a 160 day lag period was observed in Cu repleted sheep, suggesting that Cu may only be incorporated into the enzyme during erythropoeisis, and not in mature SOD enzymes containing reduced Cu levels

(Andrewartha and Caple, 1980). If Cu is not incorporated into the apo-enzyme, increased proportions of a SOD protein lacking Cu as one of the prosthetic groups may circulate within the blood.

The Cu chaperone, CCS, is involved with the movement of Cu from the cell membrane transporter, CTR1, to SOD within the cytoplasm of the cell (Fatemi and Sarkar, 2002). Work by Ogra *et al.* (1999) found TM successfully reduced SOD activity in LEC rats, similar to that of CP. These findings were suggested to be due to Cu being unavailable for incorporation into the Cu chaperones within the hepatocyte, rather than as a direct consequence of Cu being removed from the enzyme. Further work is required to elucidate the method by which TTM may alter SOD activity in ruminant animals.

Amine oxidase (AMOX) is a Cu-containing enzyme found within the plasma which has previously been shown to be relatively resistant to a primary Cu deficiency (Mills *et al.* 1976). Studies investigating AMOX activity have found that AMOX activity was inhibited in Jersey steers following intravenous injection of di and tri-TM after receiving a diet containing 25 mg kg^{-1} Mo (Mulryan and Mason, 1986). Later studies revealed inhibition of AMOX *in vitro* by di-TM (Mulryan and Mason, 1987). These studies used very high Mo concentrations, which would not normally be representative of Mo intakes *in vivo*. Therefore, chapters 3 and 6 investigated the effects of dietary Mo (which would have been representative of normal dietary Mo intakes for ruminant animals) on AMOX activity. Both chapter 3 and 6 found that AMOX activity was higher in control fed sheep compared to those animals that had received dietary Mo. Again, these findings suggest a systemic effect of TM that may reduce AMOX activity *in vivo*. The biological significance of a reduction in AMOX by TM is not known, although it is known that AMOX is important in catalysing the oxidative deamination of biogenic amines, the cross linking of collagen and regulating intracellular polyamines (Bachrach, 1985).

The homeostatic regulation of the liver in maintaining adequate Cu levels within the blood and tissues have already been discussed previously in this section. Results from studies presented in chapters 3 to 6 inclusive revealed that liver Cu concentrations were extremely sensitive to dietary Mo and Fe intakes. In all studies, dietary Mo was a more potent antagonist in reducing liver Cu concentration than dietary Fe. In chapters 3 to 6 inclusive, sheep that received the control diet maintained liver Cu concentrations considered to be within normal reference ranges for sheep (100-500 μg g^{-1} DM) (Underwood, 1977). In chapters 3, 4 and 5, dietary Mo significantly reduced liver Cu concentrations to such an extent that by the end of each respective experimental period, lambs in all three studies had liver Cu concentrations indicative of a Cu deficiency (< 100 μg g^{-1} DM).

7.4. Systemic effects of Mo or Fe on immune function

An increase in mortality and susceptibility to disease was described by Woolliams *et al.* (1986a) in lambs following grazing on improved pastures. This finding suggested a specific link between increased susceptibility to disease following exposure to dietary antagonists and the importance of Cu for effective immune function. The addition of dietary Mo or Fe in chapter 4 produced few effects on the innate and adaptive immune responses in growing lambs. As discussed previously, this may have been due to the high Cu concentration within the basal diet that lowered the proportion of dietary Mo absorbed as TM into the blood. Although significant alterations in serum CP, plasma Cu and liver Cu concentrations were observed in Chapter 4, it would seem that the systemic effect of dietary Mo or Fe were not significant enough to alter immune responses within the blood. As discussed previously, due to the lack of a suitable CD_{11a}/CD_{18a} antibody marker, the effects of dietary Mo or Fe on phagocytic function of neutrophils could not be determined. Previous studies by Gengelbach *et al.* (1997) and Boyne and Arthur (1986) found that dietary Mo reduced the killing ability of neutrophils. There were no effects of dietary Mo or Fe on the response of lymphocytes to mitogen stimulation, similar to findings in steers (Ward *et al.*, 1993) and heifers (Arthington *et al.*, 1995, 1996). The humoral immune response, as measured by antibody production, was also not affected by dietary treatment contradicting the findings of Gengelbach and Spears (1998) who found that the secondary antibody response was lower in calves supplemented with Mo.

To date, the systemic effects of absorbed TM on immune function have not been studied directly in ruminant animals by other authors. Although many authors have found conflicting effects of dietary Mo or Fe on different immune responses in ruminant animals, no firm conclusions have been stated regarding the exact mechanism by which absorbed dietary Mo (as TM) or Fe may alter specific aspects of the immune system. The effects of dietary Mo on the ruminant immune system are known to be variable and cannot be accounted for by Cu status alone (J.W. Spears, personal communication).

7.5 Systemic effects of Mo or Fe on trace element accumulation in the ovary, pituitary, cerebellum and liver

Having demonstrated that Mo and Fe produce differential effects on enzyme activities and Pl-Cu concentration, the aim of Chapter 5 was to determine if dietary Mo or Fe affected mineral retention in the ovary, pituitary, cerebellum and liver, similar to that observed due to intravenous TTM administration (Dincer, 1994; Haywood *et al.*, 2004). Results obtained from this study revealed that dietary Mo and Fe resulted in an increased mineral retention of Mo and Fe respectively within the ovary and the liver. The accumulation of Mo within the ovary, possibly due to the systemic effect of absorbed Mo as TM, may account for observed alterations in reproductive performance as described by other authors (Phillippo *et al.*, 1987b) and is discussed further in section 7.7. The systemic effect of TM may also account for the increased retention of Mo within the liver and explain the significant reductions obtained in liver Cu concentration.

Mineral retention in the pituitary gland and cerebellum was not affected by dietary treatment in this study. These findings contradict those of Haywood *et al.* (2004) who found that sheep that received ammonium TTM following Cu poisoning had elevated levels of Mo in the pituitary and in all areas of the brain. The lack of mineral accumulation in the pituitary gland in chapter 5 was surprising considering the significant alterations observed in hormone accumulation (ACTH) in the pituitary (discussed in section 7.7). The lack of effect of dietary treatment on trace element accumulation in the cerebellum may have been due to the small sample size (5 lambs per treatment) and have been too small to detect changes in mineral concentration in the brain. Further studies would require more replicates per treatment group to determine if dietary Mo and the subsequent formation of TM may cross the blood-brain barrier and accumulate in the brain. In addition, it would be interesting to determine if these minerals accumulated preferentially in other areas of the brain, in particular, the hypothalamus, which has a direct influence on the pituitary gland.

7.6.   Effect of dietary Mo or Fe on histology of the ovary and pituitary gland

Within the literature, there have been no previously known reports suggesting a possible effect of dietary Mo on histology in the ovary. In chapter 5, histopathological slides of the ovary sections could not be analysed by statistics due to the differences being morphological and not numerical (S. Haywood, Personal Communication). Results indicated a higher proportion of primordial follicles observed in the control fed lambs. These results suggested that dietary Mo may reduce the ability of new follicles to mature. In addition, a higher proportion of atretic follicles in the ovary of lambs that received dietary Mo were observed. This increased incidence of atretic follicles may have again been due to a localised toxicity effect of Mo within the ovary, causing degeneration of the follicle. Haywood *et al.* (2004) found an increased incidence of atretic follicles in sheep that received intravenous TTM. The findings in chapter 6 may further suggest that dietary Mo may alter reproductive performance in ruminant animals. This observation also adds additional information with regards to the systemic effects of absorbed Mo as TM, as also observed in the hormone release from the pituitary gland (section 7.7) and in Mo accumulation within the ovary (section 7.6).

Due to the large variation in pituitary gland histology of lambs receiving the same dietary treatment, no obvious effect of dietary treatment was concluded from this study following staining with H+E or PAS-orange G. To obtain conclusive evidence in future studies, more replicate samples would be required and a precise methodology to determine total basophil, acidophil and chromophobe populations required.

7.7.   Effect of dietary Mo or Fe on ACTH accumulation in the pituitary gland

Results obtained from chapter 5 indicate significant new evidence regarding the accumulation of the trophic hormone, ACTH, within the pituitary gland and the possible relationship between this and increased intakes of dietary Mo. There was a graduated accumulation of ACTH in pituitary tissue from lambs receiving increasing quantities of dietary Mo. Haywood *et al.* (2004) found that intravenous TTM administration to Cu-poisoned sheep resulted in retention of Mo by the pituitary gland and that pituitary glands were diminuted and of abnormal shape. The pituitary gland regulates the activity of the endocrine glands by secreting a number of different hormones that regulate growth, reproductive function and protein synthesis. ACTH is produced from corticotrophs within the anterior pituitary gland and stimulates growth of cells within the adrenal glands and regulates the secretion of glucocorticoids (Dellman, 1971). Results from chapter 5 suggest that a localised effect of absorbed dietary Mo, possibly as TM, within the pituitary may reduce the ability of the pituitary to release these hormones, in particular ACTH. The activity of the cupro-enzyme peptidylglycine α-amidating monooxygenase (PAM) has been found to be in abundance in secretory vesicles of the pituitary gland and has been found to play an important role in releasing hormones and neuropeptides from the pituitary gland (Bolkenius *et al.*, 1998). PAM is essential in the bioactivation of neuropeptides by amidating peptide precursors into their functional proteins prior to release (El Meskini *et al.*, 2003). Therefore, the observed ACTH accumulation may also suggest that other hormones produced within the anterior pituitary gland may accumulate and not be released due to the direct effect of PAM. Other hormones released from the anterior pituitary gland include luteinising hormone (LH), follicle stimulating hormone (FSH), growth hormone and thyroid stimulating hormone (TSH). LH induces ovulation in the ovarian follicles whilst FSH stimulates the development of the ovarian follicles. In studies by other authors, impaired reproductive performance and infertility in cattle has previously been associated with failure of LH release due to intakes of dietary Mo (Phillippo *et al.*, 1987b; Van

213

Niekerk and Van Niekerk, 1989b). Impaired release of LH was previously observed in heifers following intakes of dietary Mo resulting in delayed puberty and decreased conception rate (Phillippo *et al.*, 1987b). Moffor and Rodway (1991) found a decrease in pulsatile release of LH in lambs that received intravenous TTM, further suggesting that TM may alter hormone release from the pituitary. It remains unclear if reduced LH ovulatory peaks are due a direct effect of LH release from the pituitary gland or if the effect of TM is having an effect on oestrogen receptor activity as demonstrated *in vitro* by Shyamala and Leonard (1980).

Until now, it was not known how dietary Mo may affect pituitary hormone release. Intravenous administration of ammonium TTM was found to deplete ACTH-positive cells in sheep (Haywood *et al.*, 2004), a finding that contradicts those found in chapter 5. Future studies therefore need to concentrate on the systemic effect of dietary Mo and how other hormones, particularly those associated with the reproductive hormones, are directly affected by dietary Mo. More work also needs to focus on the effects of TM on PAM activity in ruminant animals, and how reduced PAM activity may alter the release of the reproductive hormones including LH and FSH.

7.8.   *Effect of Mo or Fe on ceruloplasmin expression in the liver*

Throughout this series of studies, and from results obtained by previous authors (Humphries *et al.*, 1983; Mackenzie *et al.* 1997), the activity of the cupro-enzyme, CP, has been found to be reduced by dietary Mo. Although CP is known to be produced at a constitutive rate in the liver of rats (Gitlin *et al.*, 1992), the mechanism by which the dietary antagonists Mo or Fe reduced CP activity remained unclear. Studies by Lannon and Mason (1986) found that CP could be reversibly inhibited by TM production *in vivo* and *in vitro*, and therefore, chapter 6 was undertaken to determine if dietary Mo or Fe altered the expression of CP in the liver of growing lambs. Results obtained from this study revealed that CP was expressed at a constitutive rate in the liver and was not affected by dietary treatment. Therefore, the reductions in serum CP activity observed in chapters 3 and 5 suggest that CP was either being released as the apo-protein, deficient of Cu at the active sites and lacking true oxidase activity, or that TM may systemically reduce CP activity by an as yet, unknown mechanism. TM was not directly affecting the expression of CP within the hepatocyte. This novel finding may now eliminate the possibility that CP expression is altered within the hepatocyte, and therefore, future work needs to focus on the mechanisms by which TM systemically reduces the activity of CP.

7.9. Conclusion and further work

This series of studies has produced some fundamental novel findings with respect to the antagonistic interactions of dietary Mo and Fe on Cu metabolism and physiology of sheep. Alterations in Cu in blood and tissues due to dietary Mo and Fe have been confirmed in these studies. These alterations were observed in Pl-Cu concentrations, CP activity and alterations in mineral accumulation within the liver and ovary. ACTH also accumulated in the corticotrophs of the pituitary due to increasing intakes of dietary Mo. The findings not only confirm theories and observations noted from previous authors within the literature, but have generated ideas for future studies to determine the complex antagonistic interactions of dietary Mo and Fe on Cu metabolism.

The precise mechanism by which dietary Fe alters Cu metabolism was out of the scope of this thesis but would be of interest for future studies. This work has generated ideas, particularly with respect to mechanisms in which dietary Mo may alter reproductive performance, both within the ovary and the direct influences of the pituitary gland. These important effects that would dramatically affect the performance of a ruminant animal may be ignored if Pl-Cu had been used as the sole criteria for determining Cu status. Future studies would also be beneficial in determining one reliable diagnostic test to determine the true Cu status in ruminant animals, taking into consideration the proportion of absorbed TM. Additional studies are also required to elucidate the direct effects of TM on the Cu chaperones within the hepatocyte and to determine if the systemic effect of TM is directly responsible for CP inhibition.

REFERENCES

Abumrad, N.N. 1984 Molybdenum: it is an essential trace metal. *Bulletin of New York Academy of Scientific Medicine 60* : 163-171

AFRC (Agricultural and Food Research Council) 1993 *Energy and Protein Requirements of Ruminants.* An advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients. CAB International, Wallingford, Oxon, UK

Agricultural Research Council (ARC) 1980 *The Nutrient Requirements of Ruminant Livestock.* Commonwealth Agricultural Bureau, UK

Aldred, A.R., Grimes, A., Schreiber, G. and Mercer, J.F. 1987 Rat ceruloplasmin. Molecular cloning and gene expression in liver, choroid plexus, yolk sac, placenta and testis. *Journal of Biological Chemistry 262 (6)* : 2875-2878

Alexander, J. and Brewer, J.M. 1995 Adjuvants and their mode of action. *Livestock Production Science 42* : 153-162

Allcroft, R. and Parker, W.H. 1949 Hypocupraemia in dairy cows. *British Journal of Nutrition 3* : 205

Alleyne, T., Adogwa, A., Lalla, A., Joseph, J. and John, R. 1996 Novel mitochondrial proteins and decreased intrinsic activity of cytochrome-c-oxidase. *Molecular and Chemical Neuropathology 28*: 285-293

Alleyne, T., Joseph, J., Lalla, A., Sampson, V. and Adogwa, A. 1998 Cytochrome-C oxidase isolated from the brain of swayback-diseased sheep displays uncharacteristic kinetics. *Molecular and Chemical Neuropathology 34* : 233-247

Andrewartha, K.A. and Caple, I.W. 1980 Effects of changes in nutritional copper on erythrocyte superoxide dismutase activity in sheep. *Research in Veterinary Science 28* : 101-104

AOAC (Association of Analytical Chemists) 1990 Veterinary Analytical Toxicology: Copper in Liver. Method 985.40. In: Official Methods of Analysis of AOAC International. Chapter 14, page 1

ARC (Agricultural Research Council) 1980 *The Nutrient Requirements of Ruminant Livestock.* Farnham Royal, Commonwealth Agricultural Bureaux

Armstrong, C., Leong, W. and Lees, G.J. 2001 Comparative effects of metal chelating agents on the neuronal cytotoxicity induced by copper (Cu^{2+}), iron (Fe^{3+}) and zinc in the hippocampus. *Brain Research 892* : 51-62

Arthington, J.D., Corah, L.R., Blecha, F. and Hill, D.A. 1995 Effect of copper depletion and repletion on lymphocyte blastogenesis and neutrophil bactericidal function in beef heifers. *Journal of Animal Science 73* : 2079-2085

Arthington, J.D., Corah, L.R. and Blecha, F. 1996 The effect of molybdenum-induced copper deficiency on acute-phase protein concentrations, superoxide dismutase activity, leukocyte numbers, and lymphocyte proliferation in beef heifers inoculated with bovine herpesvirus. *Journal of Animal Science 74* : 211-217

Bachrach, U. 1985 Copper amine oxidases and amines as regulators of cellular processes. In: Mondovi, B. (ed) *Structure and Functions of Amine Oxidases*. CRC Press, Boca Raton, Florida

Beck, AB. 1941 Studies on the blood copper of sheep and of cows. *Australian Journal of Experimental Biology and Medical Science 19* : 145

Beeson, K.C., Gray, L. and Adams, M.B. 1947 The absorption of mineral elements by forage plants. I. The phosphorus, cobalt, manganese and copper content of some common grasses. *Journal of the American Society of Agronomy 39* : 356-362

Bires, J., Marecek, I., Bartho, P., Biresova, M. and Weissova, T. 1995 Accumulation of trace elements in sheep and the effects upon qualitative and quantitative ovarian changes. *Veterinary Human Toxicology 37 (4)* : 349-356

Bissig, K., Voegelin, T.C. and Solioz, M. 2001 Tetrathiomolybdate inhibition of the *Enterococcus hirae* CopB copper ATPase. *Febs Letters 507* : 367-370

Black, D.H. and French, N.P. 2000 Copper supplementation and bovine pregnancy rates: three types of supplementation compared in commercial dairy herds. *Irish Veterinary Journal 53* : 213-222

Blakemore, F. and Venn, J.A.J. 1950 Conditions associated with hypocupraemia of bovine in East Anglia. *Veterinary Record 62* : 756-761

Blakley, B.R. and Hamilton, D.L. 1985 Ceruloplasmin as an indicator of copper status in cattle and sheep. *Canadian Journal of Comparative Medicine 49* : 405-408

Bolkenhuis, F.N. and Ganzhorn, A.J. 1998 Review. Peptidyl α-amidating monooxygenase: neuropeptide amidation as a target for drug design. *General Pharmacology 31* : 655-659

Bossa, F., Barra, D., Marmocchi, F., Martini, F., Rotilio, G., Bannister, J.V. and Bannister, W.H. 1980 In: Bannister, J.V. and Hill, H.H.O. (eds) *Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase*. Elsevier, New York

Boyne, R. and Arthur, J.R. 1986 Effects of molybdenum or iron induced copper deficiency on the viability and function of neutrophils from cattle. *Research in Veterinary Science 41* : 417-419

Braas, K.M., Stoffers, D.A., Eipper, B.A. and May, V. 1989 Tissue specific expression of rat peptidylglycine α-amidating monooxygenase activity and mRNA. *Molecular Endocrinology 3* : 1387-1398

Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. 1982 Mechanism of C-terminal amide formation by pituitary enzymes. *Nature 298* : 686-688

Bremner, I. 1980 Absorption, transport and distribution of copper. In: *Biological Roles of Copper*. CIBA Foundation Symposium *79* : 23-48

Bremner, I. And Dalgarno, A.C. 1973 Iron metabolism in the veal calf. 2. Iron requirements and the effect of copper supplementation. *The British Journal of Nutrition 30 (1)* : 61-76

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

Bremner, I., Young, B.W. and Mills, C.F. 1976 Protective effect of zinc supplementation against copper toxicosis in sheep. *British Journal of Nutrition 36* : 551-561

Bremner, I., Phillippo, M., Humphries, W.R., Young, B.W. and Mills, C.F. 1983 Effect of iron and molybdenum on copper metabolism in calves. In: *Trace Elements in Animal Production and Veterinary Practice*. Suttle, N.F., Gunn, R.G., Allen, W.M., Linklater, K.A. and Wiener, G. (eds) BSAP Occasional Publication No. 7.

Bremner, I., Humphries, W.R., Phillippo, M., Walker, M.J. and Morrice, P.C. 1987 Iron-induced copper deficiency in calves: dose-response relationships and interactions with molybdenum and sulphur. *Animal Production 45* : 403-414

Brewer, G.J., Dick, R.D., Johnson, Y., Wang, Y., Yuzbasiyan-Gurkan, F.K., Kluin, J.K., Fink, J.K. and Aisen, A. 1994 Treatment of Wilson's Disease with ammonium tetrathiomolybdate. I. Initial therapy in 17 neurologically affected patients. *Archives of Neurology 51* : 545-554

Bronner, F. and Yost, J.H. 1985 Saturable and non-saturable copper and calcium transport in mouse duodenum. *American Journal of Physiology 249* : G108-G112

Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schilz-Schaeffer, W., Giese, A., Westaway, D. and Kretzschmar, H. 1997 The cellular prion protein binds copper *in vivo*. *Nature 390* : 684-690

Brown, D.R., Wong, B.S., Hafiz, F., Clive, C., Haswell, S.J. and Jones, I.M. 1999 Normal prion protein has an activity like that of superoxide dismutase. *Biochemistry Journal 344* : 1-5

Buckley, W.T. 1991 A kinetic model of copper metabolism in lactating dairy cows. *Canadian Journal of Animal Science 71* : 155-166

Bull, P.C., Thomas, G.R., Rommens, J.M., Forbes, J.R. and Cox, D.C. 1993 The Wilson Disease is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nature Genetics 5* : 327-337

Bush, A.I. 2000 Metals and Neuroscience. *Current Opinions in Chemical Biology 4* : 184-191

Calabrese, L., Capuozzo, E., Galtieri, A. and Bellocco, E. 1983 Sheep ceruloplasmin: isolation and characterisation. *Molecular and Cellular Biochemistry 51* : 129-132

Cannon, H.L., Connally, G.G., Epstein, J.B., Parker, J.G., Thornton, I. and Wixson, B.G. 1978 Rocks: the geologic source of most trace elements. *Geochemistry and the Environment 3* : 17-31

Case, A.A., Selby, L.A., Hutcheson, D.P., Ebens, R.J., Erdman, J.A. and Feder, G.L. 1973 Infertility and growth suppression in beef cattle associated with abnormalities in their geochemical environment. *Trace Substances in Environmental Health VI* : 15-21

Chang, L.Y., Slot, J.W., Geuze, H.J. and Crapo, J.D. 1988 Molecular immunocytochemistry of the CuZn superoxide dismutase hepatocytes. *Journal of Cell Biology 107 (6)* : 2169-2179

Chance, B. and Leigh, J.S., Jnr. 1977 Oxygen intermediates and mixed valence states of cytochrome oxidase: Infrared absorption difference spectra of compounds A, B and C of cytochrome oxidase and oxygen. *Proceedings of the National Academy of Science, United States 74* : 4777-4780

Chase, C.R., Beede, D.K., Van Horn, H.H., Shearer, J.K., Wilcox, C.J. and Donovan, G.A. 2000 Responses of lactating dairy cows to copper source, supplementation rate and dietary antagonist (iron). *Journal of Dairy Science 83* : 1845-1852

Chidambaram, M.V., Barnes, G. and Frieden, E. 1984 Inhibition of ceruloplasmin and other copper oxidases by thiomolybdate. *Journal of Inorganic Biochemistry 22* : 231-239

Clarke, N.J. and Laurie, S.H. (1980) The copper-molybdenum antagonism in ruminants. I. The formation of thiomolybdates in animal rumen. *Journal of Inorganic Biochemistry 12* : 37-43

Clarke, N.J, Laurie, S.H. and Pratt, D.E. 1987 The copper-molybdenum antagonism in ruminants IV. Reaction of thiomolybdate ions with proteins and metalloproteins. *Inorganica Chimica Acta 138* : 103-104

Claypool, D.W., Adams, F.W., Pendell, H.W., Hartmann, N.A. and Bone, J.F. 1975 Relationship between the level of copper in the blood plasma and liver of cattle. *Journal of Animal Science 41* : 911-914

Connor, J.G., Eckersall, P.D., Wiseman, A., Aitchson, T.C. and Douglas, T.A. 1986 Bovine acute phase response following turpentine injection. *Research in Veterinary Science 44* : 82-88

Cousins, R.J. 1985 Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and caeruloplasmin. *Physiological Reviews 65 (2)* : 238-309

Cunningham, I.J. 1950 Copper and molybdenum in relation to diseases of cattle and sheep in New Zealand. In: McElroy, W.D. and Glass, B. (eds) *Symposium on Copper Metabolism.* John Hopkins Press, Baltimore

Danks, D.M. 1995 Disorders of copper transport. In: Scriver, C.R., Beaudet, A.L., Sly, W.M. and Valle, D. (eds) *The Metabolic and Molecular Basis of Inherited Disease.* McGraw-Hill, New York

Danks, D.M., Campbell, P.E., Stevens, B.J., Mayne, V. and Cartwright, E. 1972 Menkes' kinky hair syndrome. An inherited defect in copper absorption with widespread effects. *Pediatrics 50* : 188-201

Davis, G.K. and Mertz, W. 1987 In: *Trace Elements in Human and Animal Nutrition.* Mertz, W. (ed) Academic Press, London

Dellman, H. 1971 *Veterinary Histology: An Outline Text-Atlas.* Lea & Febiger, Philadelphia

Dewes, H.F. 1996 The rate of soil ingestion by dairy cows and the effect on available copper, calcium, sodium and magnesium. *New Zealand Veterinary Journal 44* : 199-200

Dick, A.T. 1953 The effect of inorganic sulphate on the excretion of molybdenum in the sheep. *Australian Veterinary Journal 29* : 18-26

Dick, A.T. 1954 Studies on the assimilation and storage of copper in crossbred sheep. *Australian Journal of Agricultural Research 5* : 511-544

Dick, A.T. 1956 Molybdenum in animal nutrition. *Soil Science 81* : 229-258

Dick, A.T., Dewey, D.W. and Gawthorne, D.M. 1975 Thiomolybdates and the copper-molybdenum-sulphur interaction in ruminant nutrition. *Journal of Agricultural Science, Cambridge 85* : 567-568

Didonato, M. and Sarkar, B. 1997 Copper transport and its alterations in Menkes and Wilsons diseases. *Biochimica et Biophysica Acta 1360 (1)* : 3 -16

Dincer, Z. 1994 Copper toxicity in sheep: studies on copper chelation by ammonium tetrathiomolybdate (TTM) and metallothionein. PhD Thesis, University of Liverpool

Du, Z., Hemken, R.W. and Harmon, R.J. 1996 Copper metabolism of Holstein and Jersey cows and heifers fed diets high in cupric sulfate or copper proteinate. *Journal of Dairy Science 79* : 1873-1880

Du Plessis, S.S., van Niekerk, F.E. and Coetzer, W.A. 1999a The effect of dietary molybdenum and sulphate on the oestrus cycle and ovulation in ewes after manipulation with exogenous progesterone alone or in combination with FSH and LH. *Small Ruminant Research 33* : 63-69

Du Plessis, S.S., van Niekerk, F.E. and Coetzer, W.A. 1999b The effect of dietary molybdenum and sulphate on sexual activity and plasma progesterone concentrations of ewes. *Small Ruminant Research 33* : 71-76

Eckersall, P.D., Duthie, S., Safi, S., Moffatt, D., Horadagoda, N.U., Doyle, S., Parton, R., Bennett, D. and Fitzpatrick, J.L. 1999 An automated biochemical assay for haptoglobin: prevention of interference from albumin. *Comparative Haematology International 9* : 117-124

Edwards, S.G., Pirgozliev, S.R., Hare, M.C. and Jenkinson, P. 2001 Quantification of Trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium Head Blight of winter wheat. *Applied and Environmental Microbiology 67 (4)* : 1575-1580

Eipper, B.A., Milgram, S.L., Husten, E.J., Yun, H-Y. and Mains, R.E. 1993 Peptidylglycine α-amidating mono-oxygenase: A multifunctional protein with catalytic, processing and routing domains. *Protein Science 2* : 489-497

El Meskini, R., Delfino, C., Boudouresque, F., Oliver, C., Martin, P.M. and Ouafik, L. 1998 Evidence of high expression of peptidylglycine α-amidating monooxygenase in the rat uterus: Estrogen regulation. *Proceeding of the National Academy of Science 95* : 7191-7196

El Meskini, R., Culotta, V.C., Mains, R. E. and Eipper, B.A. 2003 Supplying copper to the cuproenzyme peptidylglycine α-amidating monooxygenase. *The Journal of Biological Chemistry 278* (14) : 12278-12284

Evans, G.W. 1973 Copper homeostasis in the mammalian system. *Physiological Reviews 53* : 535-570

Evering, W.E., Haywood, S., Bremner, I., Wood, A.M. and Trafford, J. 1991 The protective role of metallothionein in copper overload II. Transport and excretion of immunoreactive MT-1 in blood, bile and urine of copper-loaded rats. *Chemical and Biological Interactions 78 (3)* : 297-305

Fatemi, N. and Sarkar, B. 2002 Insights into the mechanism of copper transport by the Wilson and Menkes disease copper-transporting ATPases. *Inorganica Chimica Acta 339* : 179-187

Fee, J.A. 1975 Copper proteins – Systems containing the 'blue' copper center. *Structural Bonding (Berlin)* Dunitz, J.D., Hemmerich, P., Hol, R.H., Ibers, J.A., Jorgensen, C.K., Neilands, J.B., Reinen, D. and Williams, R.J.P. (eds) Springer-Verlag, New York *23* : 1-60

Fell, B.F., Mills, C.F. and Boyne, R. 1965 Cytochrome oxidase deficiency in motor neurones of copper-deficient lambs: a histochemical study. *Research in Veterinary Science 6* : 170-177

Ferguson, W.S., Lewis, A.H. and Watson, S.J. 1943 The teart pastures of Somerset (I) The cause and cure of teartness. *Journal of Agricultural Science 33* : 44-51

Flaminio, M.J.B.F., Rush, B.R., Davis, E.G., Hennessy, K., Shuman, W. and Wilkerson, M.J. 2000 Characterization of peripheral blood and pulmonary leukocyte function in healthy foals. *Veterinary Immunology and Immunopathology 73* : 267-285

Fleming, R.E., Whitman, I.P. and Gitlin, J.D. 1991 Induction of ceruloplasmin gene expression in rat lung during inflammation and hyperoxia. *American Journal of Physiology 260 (1)* : L68-L74

Frank, A. 1998 'Mysterious' moose disease in Sweden. Similarities to copper deficiency and/or molybdenosis in cattle and sheep. Biochemical background of clinical signs and organ lesions. *Science of the Total Environment 209* : 17-26

Frank, A., Sell, D.R., Danielsson, R., Fogarty, J.F. and Monnier, V.M. 2000a A syndrome of molybdenosis, copper deficiency and type 2 diabetes in the moose population of South-West Sweden. *The Science of the Total Environment 249* : 123-131

Frank, A., Danielsson, R. and Jones, B. 2000b The 'mysterious' disease in Swedish Moose. Concentrations of trace elements in liver and kidneys and clinical chemistry. Comparison with experimental molybdenosis and copper deficiency in the goat. *The Science of the Total Environment 249* : 107-122

Frank, A., Anke, M. and Danielsson, R. 2000c Experimental copper and chromium deficiency and additional molybdenum supplementation in goats. (1) Feed consumption and weight development. *The Science of the Total Environment 249* : 133-142

Frank, A., Danielsson, R. and Jones, B. 2000d Experimental copper and chromium deficiency and additional molybdenum supplementation in goats. II. Concentrations of trace and minor elements in liver, kidneys and ribs: haematology and clinical chemistry. *The Science of the Total Environment 249* : 143-170

Freudenberger, A.S., Familton, A.S. and Sykes, A.R. 1987 Comparative aspects of copper metabolism in silage-fed sheep and deer (*Cervus elaphus*). *Journal of Agricultural Science, Cambridge 108* : 1-7

Fridovich, I. 1975 Superoxide dismutases. *Annual Reviews of Biochemistry 44* : 147-159

Frieden, E. 1970 Ceruloplasmin: a link between copper and iron metabolism. *Nutrition Reviews 28 (4)* : 87

Galbraith, H., Chigwada, W., Scaife, J.R. and Humphries, W.R. 1997 The effect of dietary molybdenum supplementation on tissue copper concentrations, mohair fibre and carcass characteristics of growing Angora goats. *Animal Feed Science and Technology 67* : 83-90

Gallagher, C.H., Reeve, V.E. and Wright, R. 1973 Copper deficiency in the rat. Effect on the ultrastructure of hepatocytes. *Australian Journal of Experimental Biology and Medical Science 51* : 181-189

Gasbarre, L.C. 1986 Limiting dilution analyses for the quantification of cellular immune responses in bovine ostertagiasis. *Veterinary Parasitology 20* : 133-147

Gengelbach, G.P. and Spears, J.W. 1998 Effects of dietary copper and molybdenum on copper status, cytokine production and humoral immune response of calves. *Journal of Dairy Science 81* : 3286-3292

Gengelbach, G.P., Ward, J.D., Spears, J.W. and Brown, T.T. jnr. 1997 Effects of copper deficiency or copper deficiency coupled with high dietary iron or molybdenum on phagocytic cell function and response of calves to a respiratory disease challenge. *Journal of Animal Science 75* : 1112-1118

Georgievskii, V.I., Annenkov, B.N. and Samokhin, V.T. 1982 *Mineral Nutrition of Animals.* Butterworths, London, UK

Gibbs, K. and Walshe, J.M. 1979 A study of the caeruloplasmin concentrations found in 75 pateints with Wilson's Disease, their kinship and various control groups. *Journal of Medicine 48 (191)* : 447-463

Gitlin, J.D., Schroeder, J.J., Lee-Ambrose, L.M. and Cousins, R.J. 1992 Mechanisms of caeruloplasmin biosynthesis in normal and copper-deficient rats. *Biochemistry Journal 282* : 835-839

Goldberg, B. and Stern, A. 1977 The role of the superoxide anion as a toxic species in the erythrocyte. *Archives of Biochemistry and Biophysiology 178* : 218-225

Goldstein, I.M. and Charo, I.F. 1982 Ceruloplasmin: An acute phase reactant and anti-oxidant. *Lymphokines 8* : 373-411

Gooneratne, S.R. and Christensen, D.A. 1984 Increased copper excretion in bile after thiomolybdate administration. *Federation Proceedings 43* : 790

Gooneratne, S.R., McC. Howell, J. and Gawthorne, J. 1979 Intracellular distribution of copper in the liver of normal and copper loaded sheep. *Research in Veterinary Science 27* : 30-37

Gooneratne, S.R., McC. Howell, J. and Gawthorne, J.M. 1981a Intravenous administration of thiomolybdate for the prevention and treatment of chronic copper poisoning in sheep. *British Journal of Nutrition 46* : 457-467

Gooneratne, S.R., McC. Howell, J. and Gawthorne, J.M. 1981b An investigation of the effects of intravenous administration of thiomolybdates on copper metabolism in chronic copper-poisoned sheep. *British Journal of Nutrition 46* : 469-480

Gooneratne, S.R., Christensen, D.A., Bailey, J.V. and Symonds, H.W. 1988 Influence of breed and dietary Cu, Mo and S levels on biliary Cu excretion in cattle. In: Hurley, L.S., Keen, C.L., Lonnerdal, B. and Rucker, R.B. (eds) *Proceedings of the Sixth Symposium on Trace Elements in Man and Animals (TEMA-6)*. Plenum Press, New York.

Gooneratne, S.R., Buckley, W.T. and Christensen, D.A. 1989a Review of copper deficiency and metabolism in ruminants. *Canadian Journal of Animal Science 69 (4)* : 819-845

Gooneratne, S.R., Chaplin, R.K., Trent, A.M. and Christensen, D.A. 1989b Effect of tetrathiomolybdate administration on the excretion of copper, zinc, iron and molybdenum in sheep bile. *British Veterinary Journal 145* : 62-72

Gooneratne, S.R., Laarveld, B., Chaplin, R.K. and Christensen, D.A. 1989c Profiles of ^{67}Cu in blood, bile, urine and faeces from ^{67}Cu-primed lambs: effects of ^{99}Mo-labelled tetrathiomolybdate on the metabolism of recently stored tissue ^{67}Cu. *British Journal of Nutrition 61* : 355-371

Gooneratne, S.R., Symonds, H.W., Bailey, J.V. and Christensen, D.A. 1994 Effects of dietary copper, molybdenum and sulphur on biliary copper and zinc excretion in Simmental and Angus cattle. *Canadian Journal of Animal Science 74*: 315-325

Grace, N.D. 1975 Studies on the flow of zinc, cobalt, copper and manganese along the digestive tract of sheep given fresh perrenial ryegrass, or white or red clover. *British Journal of Nutrition 34* : 73-82

Grace, N.D. and Lee, J. 1990 Effect of Co, Cu, Fe, Mn, Mo, Se and Zn supplementation on the elemental content of soft tissues and bone in sheep grazing ryegrass/white clover pasture. *New Zealand Journal of Agricultural Research 33* : 635-647

Grace, N.D., Rounce, J.R. and Lee, J. 1997 Effect of high sulphur intake as elemental and thiosulphate sulphur on the copper status of grazing lambs. In: Fischer, P.W.F., L'Abbé, M.R., Cockell, K.A. and Gibson, R.S. (eds) *Trace Elements in Man and Animals – 9: Proceedings of the Ninth International Symposium on Trace Elements in Man and Animals.* NRC Research Press, Ottawa, Canada.

Green, D.E., Basford, R.E. and Mackler, B. 1956 *The role of iron and copper in terminal electron transport.* In: McElroy, W.D. and Glass, B. (eds) A Symposium on Inorganic Nitrogen Metabolism: Function of metallo-falvoproteins. John Hopkins Press, Baltimore, USA

Griebel, P.J. 1998 Sheep immunology and goat peculiarities. In: Pastoret, P.P. (eds) *Handbook of vertebrate immunology.* Academic Press Ltd., London

Gubler, C.J., Lahey, M.E., Chase, M.S., Cartwright, G. and Wintrobe, M.M. 1952 Studies on copper metabolism. III. The metabolism of iron in copper deficient swine. *Blood 7* : 1075

Gubler, C.J., Cartwright, G.E. and Wintrobe, M.M. 1957 Studies on copper metabolism. XX. Enzyme activities and iron metabolism in copper and iron deficiencies. *Journal of Biological Chemistry 224* : 533-546

Harris, E.D. 1991 Copper Transport: An overview. *Proceedings of the Society of Experimental Biology and Medicine 196* : 130-140

Harris, E.D., Rayton, J.K., Balthorp, J.E., DiSilvestro R.A. and Garcia-de-Quevedo, M. 1980 Copper and the synthesis of elastin and collagen. In: *Biological Roles of Copper.* Ciba Foundation Symposium 79 : 163-182

Hart, E.B., Steenbock, H., Waddell, J. and Elvehjem, C.A. 1928 Iron in nutrition. 7. Copper as a supplement to iron for hemoglobin building in the rat. *Journal of Biological Chemistry 77* : 797-812

Hartmans, J. and Bosman, M.S.M. 1970 *Differences in the copper status of grazing and housed cattle and their biochemical backgrounds.* In: Mills, C.F. (ed) *Trace Element Metabolism in Animals.* E. & S. Livingstone, Edinburgh

Haywood, S., Dincer, Z. and Humphries, W.R. 1993 Endocrinopathy and brain copper elevation in tetrathiomolybdate treated sheep. In: M. Anke, D. Meissner and C.F. Mills (eds) *Trace Elements in Man and Animals – 8: Proceedings of the Eighth International Symposium on Trace Elements in Man and Animals, Dresden. Gersdorf: Verlag Media Touristik.*

Haywood, S. and Dincer, Z. 1997 Ammonium tetrathiomolybdate (TTM) and metal distribution in copper supplemented sheep. In: P.W.F. Fischer, M.R. L'Abbé, K.A. Cockell and R.S. Gibson. (eds) *Trace Elements in Man and Animals – 9: Proceedings of the Ninth International Symposium on Trace Elements in Man and Animals.* NRC Research Press, Ottawa, Canada.

Haywood, S., Fuentealba, I.C., Foster, J. and Ross, G. 1996 Pathiobiology of copper-induced injury in Bedlington Terriers : Ultrastructural and microanalytical studies. *Analytical Cellular Pathology 10* : 229-241

Haywood, S., Dincer, Z., Holding, J. and Parry, N.M. 1998 Metal (molybdenum, copper) accumulation and retention in brain, pituitary and other organs of ammonium tetrathiomolybdate-treated sheep. *British Journal of Nutrition 79* : 329-331

Haywood, S., Dincer, Z., Jasani, B. and Loughran, M.J. 2004 Molybdenum-associated pituitary endocrinopathy in sheep treated with Ammonium Tetrathiomolybdate. *Journal of Comparative Pathology 130* : 21-31

Henry, R.J., Cannon, D.C. and Winkelman, J.W. 1974 *Clinical Chemistry : Principles and Techniques.* Harper and Row Publishers, London

Herdt, T.H., Rumbeiha, W. and Braselton, W.E. 2000 The use of blood analyses to evaluate mineral status in livestock. *Veterinary Clinics of North America : Food Animal Practice 16 (3)* : 423-444

Hogan, K.G., Money, D.F.L., White, D.A. and Walker, R. 1971 Weight responses of young sheep to copper and connective tissue lesions associated with the grazing of pastures of high molybdenum content. *New Zealand Journal of Agricultural Research 14* : 687-701

Holmberg, C.G. and Laurell, C.B. 1948 Investigations of serum copper. II. Isolation of the copper containing protein and a description of some of its properties. *Acta Chemica Scandinavia 2* : 550-556

Holtzman, N.A. and Gaumnitz, B.M. 1970 Identification of an apoceruloplasmin-like substance in the plasma of copper-deficient rats. *Journal of Biological Chemistry 245 (9)* : 2350

Howell, J.M. and Davison, A.N. 1959 The copper content and cytochrome oxidase activity of tissues from normal and swayback lambs. *Biochemical Journal 72* : 365-368

Howell, J.M., Davison, A.N. and Oxberry, J. 1964 Biochemical and neuropathological changes in swayback. *Research in Veterinary Science 5* : 376-384

Hudson, L. and Hay, F.C. 1989 *Practical Immunology.* Third Edition. Blackwell Scientific Publications, Oxford

Humphries, W.R., Phillippo, M., Young, B.W. and Bremner, I. 1983 The influence of dietary iron and molybdenum on copper metabolism in calves. *British Journal of Nutrition 49* : 77-86

226

Humphries, W.R., Bremner, I. And Phillippo, M. 1985 The influence of dietary copper on copper metabolism in the calf. In: Mills, C.F., Bremner, I. And Chesters, J.K. (eds) *Trace Elements in Man and Animals (TEMA-5)* Commonwealth Agricultural Bureaux, Farnham Royal, UK

Humphries, W.R., Mills, C.F., Greig, A., Roberts, L., Inglis, D. and Halliday, G.J. 1986 Use of ammonium tetrathiomolybdate in the treatment of copper poisoning in sheep. *Veterinary Record 119* : 596-598

Hynes, M., Lamand, M., Montel, G. and Mason, J. 1984 Some studies on the metabolism and the effects of ^{99}Mo and ^{35}S-labelled thiomolybdates after intravenous infusion in sheep. *British Journal of Nutrition 52* : 149-158

Irwin, M.R., Poulos, P.W., Smith, B.P., Fisher, G.L. 1974 Radiology and histopathology of lameness in young cattle with secondary copper deficiency. *Journal of Comparative Pathology 84* : 611-621

Ishmael, J., Gopinath, G. and Howell, J. Mc. 1971 Experimental chronic copper toxicity in sheep. Histological and histochemical changes during the development of lesions in the liver. *Reseach in Veterinary Science 12 (4)* : 358-366

Ivan, M. and Veira, D.M. 1985 Effects of copper sulfate supplement on growth, tissue concentration, and ruminal solubilities of molybdenum and copper in sheep fed low and high molybdenum diets. *Journal of Dairy Science 68* : 891-896

Jellinger, K.A. 1999 The role of iron in neurodegeneration: prospects for pharmacotherapy of Parkinson's disease. *Drugs Aging 14* : 115-140

Jones, D.G. and Suttle, N.F. 1981 Some effects of copper deficiency on leucocyte function in sheep and cattle. *Research in Veterinary Science 31* : 151-156

Judson, G.J. and McFarlane, J.D. 1998 Mineral disorders in grazing livestock and the usefulness of soil and plant analysis in the assessment of these disorders. *Australian Journal of Experimental Agriculture 38* : 707-723

Kagi, J.H.R. 1991 Overview of metallothionein. *Methods in Enzymology 205* : 613-626

Kalmovarin, N., Friedrichs, W.E., O'Brien, H.V., Linehan, L.A., Bowman, B.H. and Yang, F. 1991 Extrahepatic expression of plasma protein genes during inflammation. *Inflammation 15 (5)* : 369-379

Katopodis, A.G., Ping, D. and May, S.W. 1990 A novel enzyme from bovine neurointermediate pituitary catalyzes dealkylation of α-hydroxyglycine derivatives, thereby functioning sequentially with peptidylglycine α-amidating monooxygenase in peptide amidation. *Biochemistry 29* : 6115-6120

Ke, Y. and Symonds, H.W. 1987 The effect of molybdenum compounds on the biliary excretion of copper by sheep. *Proceedings of the Nutrition Society, 46* : 69a

Ke, Y. and Symonds, H.W. 1989 Enhancement of tetrathiomolybdate-induced biliary copper excretion in sheep by general anaesthaesia and the effect on copper excretion in urine and bile. *Research in Veterinary Science 46:* 344-348

Kelleher, C.A and Mason, J. 1979 The effect of tetrathiomolybdate upon sheep caeruloplasmin amine oxidase activity *in vitro* : the influence of substrate on apparent sensitivity to inhibition. *Research in Veterinary Science 26* : 124-125

Kelleher, C.A. and Ivan, M. 1986 Liver distribution of copper and ^{99}Mo in sheep following intravenous administration of copper sulfate and [^{99}Mo]-tetrathiomolybdate *Canadian Journal of Animal Science 66* : 563-565

Kelleher, C.A. and Mason, J. 1986 Reversible inhibition of ovine ceruloplasmin by thiomolybdates. *International Journal of Biochemistry 18 (7)* : 629-635

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

Kendall, N.R., Illingworth, D.V. and Telfer, S.B. 2001 Copper responsive infertility in British cattle: the use of a blood caeruloplasmin to copper ratio in determining a requirement for copper supplementation. In: Diskin, M.G. (ed) *Fertility in the High-Producing Dairy Cow.* Occasional Publication No. 26 (2) *British Society of Animal Science, Edinburgh*

Kendall, N.R., Masters, P., Scaramuzzi, R.J. and Campbell, B.K. 2003 The expression of lysyl oxidase and effect of copper chloride and ammonium tetrathiomolybdate on bovine ovarian follicle granulosa cells cultured in serum free media. *Reproduction 15* : 657-665

Kerr, M.G. 2002 The Red Blood Cells. In: *Veterinary Laboratory Medicine.* Second Edition. Blackwell Science, Oxford, UK

Kodama, H. 1993 Recent developments in Menkes Disease. *Journal of Inherited and Metabolic Disorders 16* : 791-799

Komatsu, Y., Sadakata, I., Ogra, Y. and Suzuki, K.T. 2000 Excretion of copper complexed with thiomolybdate into the bile and blood in LEC rats. *Chemico-Biological Interactions 124* : 217-231

Lal, S. and Sourkes, T.L. 1971 Intracellular distribution of copper in the liver during chronic administration. *Toxicology and Applied Pharmacology 18 (3)* : 562-572

Lannon, B. and Mason, J. 1986 The inhibition of bovine ceruloplasmin oxidase activity by thiomolybdates *in vivo* and *in vitro*: A reversible interaction. *Journal of Inorganic Biochemistry 26* : 107-115

Laurie, S.H. and Pratt, D.E. 1986 Copper-albumin: What is its functional role ? *Biochemical and Biophysical Research Communications 135 (3)* : 1064-1068

Lee, J. and Grace, N.D. 1997 A New Zealand perspective on copper, molybdenum and sulphur interactions in ruminants. In: *Proceedings from the 27th Seminar of the Society of Sheep and Beef Cattle Veterinarians, Publication no. 175.* The Society of Sheep and Beef Cattle Veterinarians. Massey University, Palmerston North, New Zealand

228

Lee, J., Knowles, S.O. and Judson, G.J. 2002 Trace element and vitamin nutrition of grazing sheep. In: Freer, M. and Dove, H. (eds) *Sheep Nutrition*. CAB International, Wallingford, UK

Lewis, D. 1954 The reduction of sulphate in the rumen of sheep. *Biochemical Journal 56* : 391-399

Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J-H., Takeichi, N., Kobayashi, H., Kojima, Y., Une, Y. and Uchino, J. 1991 Spontaneous hepatic copper accumulation in Long-Evans Cinnamon rats with hereditary hepatitis. *Journal of Clinical Investigation 87* : 1858-1861

Linder, M.C. 1991 *The Biochemistry of Copper*. Plenum Press, New York

Linder, M.C. and Hazegh-Azam, M. 1996 Copper biochemistry and molecular biology. *American Journal of Clinical Nutrition 63* : 797S-811S

Linder, M.C., Wooten, L., Cerveza, P., Cotton, S., Shulze, R. and Lomeli, N. 1998 Copper Transport. *American Journal of Clinical Nutrition 67(5)* : 965S-971S

Littlejohn, A.I. and Lewis, G. 1960 Experimental studies of the relationship between calcium:phosphorus ratio of the diet and fertility in heifers: a preliminary report. *Veterinary Record 72*: 1137-1144

Llanos, R.M. and Mercer, J.F.B. 2002 The molecular basis of copper homeostasis and copper-related disorders. *DNA and Cell Biology 21 (4)* : 259-270

Lockhart, P.J. and Mercer, J.F. 1999 Cloning and expression analysis of the sheep ceruloplasmin cDNA. *Gene 236 (2)* : 251-257

Lockhart, P.J. and Mercer, J.F.B. 2000a Identification of the copper chaperone 'SAH' in *Ovis Aries* : expression analysis and *in vitro* interaction of SAH with ATP7B. *Biochimica et Biophysica Acta 1490* : 11-20

Lockhart, P.J., Wilcox, S.A., Dahl, H.M. and Mercer, J.F.B. 2000b Cloning, mapping and gene expression analysis of the sheep Wilson Disease gene homologue. *Biochemica et Biophysica Acta 1491* : 229-239

Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L. and Campbell, W.R. 1998 Copper, iron and zinc in Alzheimer's disease in senile plaques. *Journal of Neurological Science 158* : 47-52

Lynch, R.E., Lee, R. and Cartwright, G.E. 1976 Inhibition by superoxide dismutase of methemoglobin formation from oxyhemoglobin. *Journal of Biological Chemistry 251 (4)* : 1015-1019

Mackenzie, A.M., Illingworth, D.V., Jackson, D.W. and Telfer, S.B. 1997 The use of caeruloplasmin activities and plasma copper concentrations as an indicator of copper status in ruminants. In: Fischer, P.W.F, L'Abbe, M.R., Cockel, K.A. and Gibson, R.S. (eds) *Trace Elements in Man and Animals -9* : Proceedings of the Ninth International Symposium on Trace Elements in Man and Animals. Ottawa, Canada

Mackenzie, A.M., Moeini, M.M. and Telfer, S.B. 2001 The effect of a copper, cobalt and selenium bolus on fertility and trace element status of dairy cattle. In: Diskin, M.G. (ed) *Fertility in the High-Producing Dairy Cow.* Occasional Publication No. 26 (2) *British Society of Animal Science, Edinburgh*

Macpherson, A. 2000 Trace mineral status of forages. In: Givens, D.J., Owen, E., Axford, R.F.E. and Ohmed, H.M. (eds) *Forage Evaluation in Ruminant Nutrition.* CAB International, Wallingford, UK

MAFF 1986 The analysis of agricultural materials. Third Edition. A manual of the analytical methods used by the agricultural development and advisory service. Reference book 427.

MAFF 1992 *Feed Composition. UK Tables of Nutritive Value and Chemical Composition of Feed stuffs.* Givens, D.I. (ed) Rowett Research Services, Aberdeen, UK

Mann, T. and Keilin, D. 1938 Haemocuprein and hepatocuprein, copper-protein compounds of blood and liver in mammals. *Proceedings of the Royal Society, London 126* : 303-315

Marcilese, N.A., Ammerman, C.B., Valsecchi, R.M., Dunavant, B.G. and Davis, G.K. 1969 Effect of dietary molybdenum and sulphate on copper metabolism in sheep. *Journal of Nutrition 99* : 177

Marston, H.R. 1946 Nutrition and wool production. In: *Proceedings of a Symposium on Fibrous Proteins.* Society of Dyers and Colourists, Leeds, UK

Mason, J. 1982 The putative role of thiomolybdates in the pathogenesis of Mo-induced hypocupraemia and molybdenosis: some recent developments. *Irish Veterinary Journal 36* : 164-168

Mason, J. 1986 Thiomolybdates: Mediators of molybdenum toxicity and enzyme inhibitors. *Toxicology 42* : 99-109

Mason, J. 1990 The biochemical pathogenesis of molybdenum-induced copper deficiency syndromes in ruminants: towards the final chapter. *Irish Veterinary Journal 43* : 18-21

Mason, J., Lamand, M. and Kelleher, C.A. 1980 The fate of [99]Mo labelled sodium tetrathiomolybdate after duodenal administration in sheep: the effect on ceruloplasmin (E.C. 1.16.3.1.) diamine oxidase activity and plasma copper. *British Journal of Nutrition 43* : 515-523

Mason, J., Kelleher, C.A. and Letters, J. 1982 The demonstration of protein-bound [99]Mo-di- and trithiomolybdate in sheep plasma after the infusion of [99]Mo-labelled molybdate into the rumen. *British Journal of Nutrition 48* : 391-397

Mason, J., Woods, M. and Poole, D.B.R. 1986 Accumulation of copper on albumin in bovine plasma in vivo after intravenous trithiomolybdate administration. *Research in Veterinary Science 41* : 108-116

Mason, J., Lamand, M., Tressol, J.C. and Mulryan, G. 1988 Studies of the changes in systemic copper metabolism and excretion produced by the intravenous administration of trithiomolybdate in sheep. *British Journal of Nutrition 59(2)* : 289-300

McCord, J.M. and Fridovich, I. 1969 Superoxide dismutase: an enzymic function of erythrocuprein. *Journal of Biological Chemistry 244* : 6049-6055

McCurnin, D.M. and Bassert, J.M. 2002 *Clinical Textbook for Veterinary Technicians.* W.B. Saunders and Company, London, UK

McDonald, P., Edwards, R.A., Greenhalgh, J.F.D. and Morgan, C.A. 1995 *Animal Nutrition.* Fifth Edition. Addison Wesley Longman, Essex, UK

McDowell, L.R. 1985 Copper, molybdenum and sulfur. In: *Nutrition of grazing ruminants in warm climates.* Academic Press Ltd., New York

McDowell, L.R. 1992 Copper and Molybdenum. In: Cunha, T.J. (ed) *Minerals in Animal and Human Nutrition.* Academic Press Ltd., London

McFarlane, J.D., Judson, G.J. and Gouzos, J. 1990 Copper deficiency in ruminants in the South East of South Australia. *Australian Journal of Experimental Agriculture 30* : 187-193

McQuaid, A. and Mason, J. 1990 A comparison of the effects of pencillamine, trientine and trithiomolybdate on [^{35}S]-labelled metallothionein *in vitro*; implications for Wilson's Disease therapy. *Journal of Inorganic Biochemistry 41 (2)* : 87-92

McTaggart, C., Yovich, J.V., Penhale, J. and Raidal, S.L. 2001 A comparison of foal and adult horse neutrophil function using flow cytometric techniques. *Research in Veterinary Science 71* : 73-79

Mengal, K. and Kirkby, E.A. 1987 *Principles of Plant Nutrition.* Fourth Edition. International Potash Institute, Bern.

Menkes, J.H., Alter, M., Steigleder, G.K., Weakley, D.R. and Sung, J.H. 1962 A sex linked recessive disorder with retardation of growth, peculiar hair and focal cerebral and cerebellar degeneration. *Pediatrics 29* : 764-779

Mercer, J.F.B. 1997 Gene regulation by copper and the basis for copper homeostasis. *Nutrition 13 (1)* : 48-49

Mercer, J.F.B. 2001 The molecular basis of copper-transport diseases. *Trends in Molecular Medicine 7 (2)* : 64-69

Mills, C.F. and Williams, R.B. 1962 Copper concentration and cytochrome-oxidase and ribonuclease activities in the brains of copper-deficient lambs. *Biochemistry Journal 85* : 629-632

Mills, C.F. and Davis, G.K. 1987 Molybdenum. In: Mertz (ed) *Trace Elements in Human and Animal Nutrition.* Academic Press, New York

Mills, C.F. and Dalgarno, A.C. 1970 An evaluation of tissue cytochrome oxidase activity as an indicator of copper status. In: Mills, C.F. (ed) *Trace Element Metabolism in Animals.* E & S Livingstone, Edinburgh

Mills, C.F. and Williams, R.B. 1962 Copper concentrations and cytochrome-oxidase and ribonuclease activities in the brains of copper-deficient lambs. *Biochemistry Journal 85* : 629-632

Mills, C.F., Dalgarno, A.C. and Wenham, G. 1976 Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency. *British Journal of Nutrition 35 (3)* : 309-311

Mills, C.F., Bremner, I., El-Gallad, T.T., Dalgarno, A.C. and Young, B.W. 1978 Mechanisms of the molybdenum/sulphur anatgonism of copper utilization by ruminants. In: Kirchgessner, M. (ed) *Proceedings of the 3rd Symposium of Trace Element Metabolism in Man and Animals (TEMA-3)* Arbeitskreis fur Tierrhnarhrungsforschung, Weihenstephan, Freising, F.R.G.

Minatel, L. and Carfagnini, J.C. 2002 Evaluation of the diagnostic value of plasma copper levels in cattle. *Preventive Veterinary Medicine 53* : 1-5

Misra, H.P. and Fridovich, I. 1977 Superoxide dismutase: a photochemical augmentation assay. *Archives of Biochemistry and Biophysiology 181* : 308-312

Moeini, M.M. 1997 The effect of interactions between copper, molybdenum, iron and sulphur on metabolism and fertility in ruminant animals. PhD thesis, University of Leeds, UK

Moffor, F.M. and Rodway, R.G. 1991 The effect of tetrathiomolybdate on growth rate and onset of puberty in ewe-lambs. *British Veterinary Journal 147* : 421-431

Møller, L.B., Petersen, C., Lund, C. and Horn, N. 2000 Characterisation of the hCTR1 gene: Genomic organisation, functional expression and identification of a highly homologous processed gene. *Gene 257 (1)* : 13-22

Mosmann, T. 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods 65* : 55

Müller,T., Van de Sluis, B., Zhernakova, A., Van Binsbergen, E., Janecke, A.R., Bavdekar, A., Pandit, A., Weirich-Schwaiger, H., Wit, H., Ellemunter, H., Deutsch, J., Denk, H., Müller, W., Sternlieb, I., Tanner, M.S. and Wijmenga, C. 2003 The canine copper toxicosis gene *MURR1* does not cause non-Wilsonian hepatic copper toxicosis. *Journal of Hepatology 38 (2)* : 164-168

Mullis, L.A., Spears, J.W. and McCraw, R.L. 2003 Estimated copper requirements of Angus and Simmental heifers. *Journal of Animal Science 81* : 865-873

Mulryan, G. and Mason, J. 1987 The inhibition of bovine serum amine oxidase by thiomolybdates *in vivo* and *in vitro. Biochemical Society Transactions 15 (2)* : 248-249

Mulryan, G. and Mason, J. 1992 Assessment of liver copper status in cattle from plasma copper and plasma copper enzymes. *Annales de Recherches Veterinaires 2 (3)* : 233-238

Mulryan, G. and Mason, J. 1993 Bovine serum amine oxidase: some differences between chromatographically defined forms including sensitivity to inhibition by dithiomolybdate. *Comparative Biochemistry and Physiology 104* : 669-673

Munro, I.B. 1957 Infectious and non-infectious herd infertility in East Anglia. *Veterinary Record 69* : 125-129

Nakamura, K., Endo, F., Ueno, T., Awata, H., Tanoue, A. and Matsuda, I. 1995 Excess copper and ceruloplasmin biosynthesis in long-term cultured hepatocytes from Long-Evans Cinnamon (LEC) rats, a model of Wilson Disease. *Journal of Biological Chemistry 270 (13)* : 7656-7660

National Research Council (NRC) 1985 *Nutrient Requirements of Sheep.* Sixth Edition. National Academy Press, Washington DC, USA

O'Dell, B.L. 1976 Biochemistry of Copper. *Medicine Clinical of North America 60 (4)* : 687-703

O'Dell, B.L. and Sunde, R.A. 1997 Introduction. In: O'Dell, B.L. and Sunde, R.A. (eds) *Handbook of Nutritionally Essential Mineral Elements.* Marcel Dekker Inc., New York

O'Gorman, J., Poole, D.B.R., Boland, M.P. and Roche, J.F. 1987 The effect of molybdenum-induced copper deficiency on reproduction in beef heifers. *Theriogenology 27(1)* : 265

Oda, T. and Maeda, H. 1986 A new simple fluorometric assay for phagocytosis. *Journal of Immunological Methods 88* : 175-183

Ogra, Y., Ohmichi, M. and Suzuki, K.T. 1996 Mechanisms of selective copper removal by tetrathiomolybdatefrom metallothionein in LEC rats. *Toxicology 106* : 75-83

Ogra, Y., Komada, Y. and Suzuki, K.T. 1999 Comparative mechanism and toxicity of tetra- and dithiomolybdates in the removal of copper. *Journal of Inorganic Biochemistry 75* : 199-204

Ogra, Y., Chikusa, H. and Suzuki, K.T. 2000 Metabolic fate of the insoluble copper/tetrathiomolybdate complex formed in the liver of LEC rats with excess tetrathiomolybdate. *Journal of Inorganic Biochemsitry 78* : 123-128

Okunuki, K., Sekuzu, I., Yonetani, T. and Takamori, S. 1958 Studies on cytochrome *a.* I. Extraction, purification and some properties of cytochrome *a. Journal of Biochemistry 45* : 847-854

Owen, C.A. and Hazelrig, J.B. 1963 Copper deficiency and copper toxicity in the rat. *American Journal of Physiology 215* : 334

Pais, I. and Jones, J.B. 1997 *The Handbook of Trace Elements.* St Lucie Press, Boca Raton, Florida

Parry, N.M.A., Phillippo, M., Reid, M.D., McGraw, B.A., Flint, D.J. and Loveridge, N. 1993 Molybdenum-induced changes in the epiphyseal growth plate. *Calcified Tissue International 53 (3)* : 180-186

Paynter, D.I. 1987 The diagnosis of copper insufficiency. In: McHowell, J. and Gawthorne, J.M. (eds) *Copper in Animals and Man.* CRC Press, Boca Raton, Florida :101-119

Phillippo, M. 1983 The role of dose-response trials in predicting trace element deficiency disorders. In: *Trace Elements in Animal Production and Veterinary Practice.* Suttle, N.F., Gunn, R.G., Allen, W.M., Linklater, K.A. and Wiener, G. Occasional Publication of the British Society of Animal Production.

Phillippo, M., Humphries, W.R., Lawrence, C.B. and Price, J. 1982 Investigation of the effect of copper status and therapy on fertility in beef suckler herds. *Journal of Agricultural Science, Cambridge 99* : 359-364

Phillippo, M., Humphries, W.R. and Garthwaite, P.H. 1987a The effect of dietary molybdenum and iron on copper status and growth in cattle. *Journal of Agricultural Science Cambridge 109* : 315-320

Phillippo, M., Humphries, W.R., Atkinson, T., Henderson, G.D. and Garthwaite, P.H. 1987b The effect of dietary molybdenum and iron on copper status, puberty, fertility and oestrus cycles in cattle. *Journal of Agricultural Science 109* : 321-336

Pollock, J.M., Rowan, T.G., Dixon, J.B., Carter, S.D. and Kelly, D.F. 1991 Estimation of immunity in the developing calf: cellular and humoral responses to keyhole limpet haemocyanin. *Veterinary Immunology and Immunopathology 29* : 105-113

Pollock, J.M., McNair, J., Kennedy, S., Kennedy, D.G., Walsh, D.M., Goodall, E.A., Mackie, D. P. and Crockard, A.D. 1994 Effects of dietary vitamin E and selenium on in-vitro cellular immune responses in cattle. *Research in Veterinary Science 56* : 100-107

Pott, E.B., Henry, P.R., Rao, P.V., Hinderberger, E.J. and Ammerman, C.B. 1999a Estimated relative bioavailability of supplemental inorganic molybdenum sources and their effect on tissue molybdenum and copper concentrations in lambs. *Animal Feed Science and Technology 79* : 107-117

Practical Biochemistry 1994 Wilson, K. and Walker, J. (eds) Fourth Edition. Cambridge University Press

Price, J., Will, A.M., Paschaleris, G. and Chesters, J.K. 1987 Identification of thiomolybdates in digesta and plasma from sheep after administration of ^{99}Mo-labelled compounds in the rumen. *British Journal of Nutrition 58* : 127-138

Prohaska, J.R. 1990 Biochemical changes in copper deficiency. *Journal of Nutritional Biochemistry 1* : 452-461

Rauch, H. 1984 Toxic milk, a new mutation affecting copper metabolism in the mouse. *Journal of Hereditary 74* : 141-144

Rehbinder, C. and Petersson, L. 1994 Cerebellar abiotrophy in a moose (*Alces alces*) related to copper deficiency – a case report. *Acta Veterinaria Scandinavica 35 (1)* : 103-106

Rigo, A.,Viglino, P., Bonori, M., Cocco, D., Calabrese, L. and Rotilio, G. 1978 The binding of copper ions to copper free bovine superoxide dismutase. Kinetic aspects. *Biochemistry Journal 169* : 277-280

Roitt, I., Brostoff, J. and Male, D. 1998 *Immunology.* Fifth Edition. Mosby International Ltd., UK

Rosenzweig, A.C. 2002 Metallochaperones: Bind and Deliver. *Chemistry and Biology 9* : *673-677*

Royal, M.D., Darwash, A.O., Flint, A.P.E., Webb, R., Woolliams, J.A. and Lamming, G.E. 2000 Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. *Animal Science 70* : 487-501

Rucker, R., Romero-Chapman, N., Wong, T., Lee, J., Steinberg, F.,McGee, C., Clegg, M., Reiser, K., Kosonen, T., Urie-Hare, J., Murphy, J. and Keen, C. 1996 Modulation of lysyl oxidase by dietary copper in rats. *Journal of Nutrition 126* : 51-60

Rucker, R.B., Kosonen, T., Clegg, M.S., Mitchell, A.E., Rucker, B.R., Urie-Hare, J.Y. and Keen, C.L. 1998 Copper, lysyl oxidase and extracellular matrix protein cross-linking. *American Journal of Clinical Nutrition 67* : 996S-1002S

Saenko, E.L., Yaropolov, A.I. and Harris, E.D. 1994 Biological functions of ceruloplasmin expressed through copper-binding sites and a cellular receptor. *The Journal of Trace Elements in Experimental Medicine 7* : 69-88

Sansinanea, A.S., cerone, S.I., Streitenberger, S.A., Garcia, C. and Auza, N. 1997 Superoxide dismutase activity and reduced glutathione levels in Cu-overload liver from sheep. *Nutrition Research 17 (1)* : 115-123

Sarkar, B. 1999 Treatment of Wilson and Menkes diseases. *Chemistry Reviews 99 (9)* : 2535-2544

Sato, M. and Gitlin, J.D. 1991 Mechanisms of copper incorporation during the biosynthesis of human caeruloplasmin. *The Journal of Biological Chemistry 266 (8)* : 5128-5134

Sato, M., Hachiya, N., Yamaguchi, Y., Kubota, J., Saito, Y., Fujioka, Y., Shimatake, H., Takizawa, Y. and Aoki, T. 1993 Deficiency of holo- but not apo- ceruloplasmin in genetically copper intoxicated LEC mutant rat. *Life Science 53 (18)* : 1411-1416

Schaefer, M. and Gitlin, J.D. 1999 Genetic disorders of membrane transport (IV) Wilson's Disease and Menkes Disease. *American Journal of Physiology 276(2)* : G311-G314

Schaefer, M., Hopkins, R.G., Failla, M.L. and Gitlin, J.D. 1999 Hepatocyte specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. *American Journal of Physiology 276 (3)* : G639-G646

Scheinberg, I.H. and Gitlin, D. 1952 Deficiency of ceruloplasmin in patients with hepatolenticular degeneration (Wilson's Disease). *Science 116* : 484-485

235

Schilsky, M.L. and Sternlieb, I. 1993 Animal models of copper toxicosis. *Advances in veterinary Science and Comparative Medicine 37* : 357-377

Serazin-Leroy, V., Denis-Henriot, D., Morot, M., de Mazancourt, P. and Giudicelli, Y. 1998 Semi-quantitative RT-PCR for comparison of mRNAs in cells with different amounts of housekeeping gene transcripts. *Molecular and Cellular Probes 12 (5)* : 283-291

Shyamala, G. and Leonard, L. 1980 Inhibition of uterine estrogen receptor transformation by sodium molybdate. *Journal of Biological Chemistry 255 (13)* : 6028-6031

Sies, H. 1993 Strategies of antioxidant defence. *European Journal of Biochemistry 215* : 213-219

Slot, J.W., Geuze, H.J., Freeman, B.A. and Crapo, J.D. 1986 Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. *Laboratory Investigations 55 (3)* : 363-371

Smith, G.M. and White, C.L. 1997 A molybdenum-sulfur-cadmium interaction in sheep. *Australian Journal of Agricultural Research 48* : 147-154

Smith, B.P., Fisher, G.L., Poulos, P.W. and Irwin, M.R. 1975 Abnormal bone development and lameness associated with secondary copper deficiency in young cattle. *Journal of the American Veterinary Medical Association 166* : 682-688

Smith, R.M., Osborne-White, W.S. and O'Dell, B.L. 1976 Cytochromes in brain mitochondria from lambs with enzootic ataxia. *Journal of Neurochemistry 26* : 1145-1148

Snedecor, G.W. and Cochran, W.G. 1989 *Statistical Methods.* 8th Edition. Iowa State University Press, Ames, Iowa, USA

Sorenson, J.R.J. 2001 Prion diseases: copper deficiency states associated with impaired nitrogen monoxide or carbon monoxide transduction and translocation. *Journal of Inorganic Biochemistry 87* : 125-127

Spais, A.G., Lazaridis, T.K. and Agiannidis, A.K. 1968 Studies on sulphur metabolism in sheep in association with copper deficiency. *Research in Veterinary Science 9* : 337-344

Spears, J.W. 1999 Re-evaluation of the metabolic essentiality of the minerals – review. *Asian-Australasian Journal of Animal Sciences 12 (6)* : 1002-1008

Stabel., J.R. and Spears, J.W. 1989 Effect of copper on immune function and disease resistance. *Advances in Experimental Medicine and Biology 258* : 243-252

Stabel, J.R., Spears, J.W. and Brown, T.T. 1993 Effect of copper deficiency on tissue, blood characteristics and immune function of calves challenged with infectious bovine rhinotracheitis virus and *Pasteurella hemolytica. Journal of Animal Science 71* : 1247-1255

Standish, J.F., Ammerman, C.B., Simpson, C.F., Neal, F.C. and Palmer, A.Z. 1969 Influence of graded levels of dietary iron, as ferrous sulphate, on performance and tissue mineral composition of steers. *Journal of Animal Science 29* : 496-503

Sternlieb, I. 1993 Wilsons Disease: Extrahepatic manifestations in liver diseases. *Falk Symposium 69* : 331-336

Suttle, N.F. 1974a Effects of organic and inorganic sulphur on the availability of dietary copper to sheep. *British Journal of Nutrition 32* : 559-568

Suttle, N.F. 1974b Recent studies of the copper-molybdenum antagonism. *Nutrition Society 33* : 299

Suttle, N.F. 1975 The role of organic sulphur in the copper-molybdenum-sulphur interrelationship in ruminant nutrition. *British Journal of Nutrition 34* : 411

Suttle, N.F. 1980 The role of thiomolybdates in the nutritional interactions of copper, molybdenum and sulfur: fact or fantasy ? *Annals of the New York Academy of Science* : 195

Suttle, N.F. 1983a Assessing the mineral and trace element status of feeds. In: Robards, G.E. and Packman, R.G. (eds) *Proceedings of the Second Symposium of the International Network of Feed Information Centres.* Commonwealth Agricultural Bureaux, Farnham Royal, UK

Suttle, N.F. 1983b Effects of molybdenum concentration in fresh herbage, hay and semi-purified diets on the copper metabolism of sheep. *Journal of Agricultural Science, Cambridge 100* : 651-656

Suttle, N.F. 1986 Copper deficiency in ruminants; recent developments. *The Veterinary Record 119* : 519-522

Suttle, N.F. 1991 The interactions between copper, molybdenum and sulphur in ruminant nutrition. *Annual Review of Nutrition 11* : 121-140.

Suttle, N.F. and Angus, K.W. 1976 Experimental copper deficiency in the calf. *Journal of Comparative Pathology 86* : 595-608

Suttle, N.F. and Angus, K.W. 1978 Effects of experimental copper deficiency on the skeleton of the calf. *Journal of Comparative Pathology 88* : 135-145

Suttle, N.F. and Field, A.C. 1968 Effect of intake of copper, molybdenum and sulphate on copper metabolism in sheep: II. Copper status of the newborn lamb. *Journal of Comparative Pathology 78* : 363-370

Suttle, N.F. and Field, A.C. 1969 Effect of copper, molybdenum and sulphate on copper metabolism in sheep. 4. Production of congenital and delayed swyaback. *Journal of Comparative Pathology 79* : 453-464

Suttle, N.F. and Field, A.C. 1983 Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. *Journal of Comparative Pathology 93(3)* : 379-389

Suttle, N.F. and Jones, D.G. 1986 Copper and disease resistance in sheep: a rare natural confirmation of interaction between a specific nutrient and infection. *Proceedings of the Nutrition Society 45* : 317-325

Suttle, N.F. and Jones, D.G 1989 Recent developments in Trace Element Metabolism and Function: Trace elements, disease resistance and immune responsiveness in ruminants (Symposium) *American Institute of Nutrition* : 1055-1061

Suttle, N.F. and McMurray, C.H. 1983 Use of eryhrocyte copper:zinc superoxide dismutase activity and hair or fleece copper concentrations in the diagnosis of hypocuprosis in ruminants. *Research in Veterinary Science 35* : 47-52

Suttle, N.F. and Peter, D.W. 1985 Rumen sulphide metabolism as a major determinant of the availability of copper to ruminants. In: Mills, C.F., Bremner, I., Chesters, J.K. (eds) *Proceedings of the Fifth International Symposium on Trace Elements in Man and Animals, Aberdeen.* Commonwealth Agricultural Bureaux, Farnham Royal, UK

Suttle, N.F., Field, A.C. and Barlow, R.M. 1970 Experimental copper deficiency in sheep. *Journal of Comparative Pathology 80* : 151-162

Suttle, N.F., Angus, K.W., Nisbet, D.I. and Field, A.C. 1972 Osteoporosis in copper-depleted lambs. *Journal of Comparative Pathology 82* : 93-97

Suttle, N.F., Alloway, J. and Thornton, I. 1975 An effect of soil ingestion on the utilization of dietary copper by sheep. *Journal of Agricultural Science, Cambridge 84* : 249-254

Suttle, N.F., Abrahams, P.W. and Thornton, I. 1982 The importance of soil type and dietary sulphur in the impairment of copper absorption in sheep which ingest soil. *Proceedings of the Nutrition Society 41* : 83A

Suttle, N.F., Abrahams, P. and Thornton, L. 1984 The role of a soil x dietary sulphur interaction in the impairment of copper absorption by ingested soil in sheep. *Journal of Agricultural Science, Cambridge 103* : 81-86

Symonds, H.W and Ke, Y. 1989 Enhancement of tetrathiomolybdate-induced copper excretion of bile of sheep by the ∝-agonistic action of xylazine. *Research in Veterinary Science 46* : 349-353

Symonds, H.W. and Forbes, J. M. 1993 Mineral metabolism. In: Forbes, J.M. and France, J. (eds) *Quantitative aspects of Ruminant Digestion and Metabolism.* CAB International, Wallingford, Oxon, UK

Takahashi, N., Ortel, T.L. and Putman, F.W. 1984 *Proceedings of the National Academy of Sciences 81* : 390-394

Terada, K., Kawarada, Y., Miura, N., Yasui, O., Koyama, K. and Sugiyama, T. 1995 Copper incorporation into caeruloplasmin in rat livers. *Biochemica et Biophysica Acta 1270* : 58-62

Thomas, T., Macpherson, A. and Rogers, P. 1995 Ceruloplasmin gene expression in the rat uterus. *Biochimica et Biophysica Acta 1261 (1)* : 77-82

238

Thompson, K.G., Audige, L., Arthur, D.G., Julian, A.F., Orr, M.B., McSporran, K.D. and Wilson, P.R. 1994 Osteochondrosis associated with copper deficiency in young farmed red deer and wapiti X red deer hybrids. *New Zealand Veterinary Journal 42* : 137-143

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research 22* : 4673-4680

Thornberg, L.P., Shaw, D., Dollan, M., Raisbeck, M., Crawford, S., Dennis, G.L. and Olwin, D.B. 1986 Hereditary copper toxicosis in West Highland white terriers. *Veterinary Pathology 23* : 148-154

Thornton, I. 1983 Soil-plant-animal interactions in relation to the incidence of trace element disorders in grazing livestock. In: Suttle, N.F., Gunn, R.G., Allen, W.M., Linklater, K.A. and Wiener, G. (eds) *Trace Elements in Animal Production and Veterinary Practice.* D. & J. Croal Ltd, Haddington, UK

Tran, T., Ashraf, M., Jones, L., Westbrook, T., Lo, L., Liu, N., Williams, P. and Linder, M.C. (1999) Potential role of ceruloplasmin in iron transport: effects of nutritional iron and copper status on expression of selected iron and copper proteins. *Journal of Inorganic Biochemistry 74* : 71

Turnland, J.R. 1998 Human whole-body copper metabolism. *American Journal of Clinical Nutrition 67* : 960S-964S

Underwood, E.J. 1977 *Trace elements in Human and Animal Nutrition.* Fourth Edition. Academic Press, New York.

Underwood, E.J. and Suttle, N.F. 1999 *The Mineral Nutrition of Livestock.* CABI International, Wallingford, UK

Van der Sluis, B., Rothuizen, J., Pearson, P.L., van Oost, B.A. and Wijmenga, C. 2002 Identification of a new copper metabolism gene by positional cloning a purebred dog population. *Human Molecular Genetics 11 (2)* : 165-173

Van Niekerk, F.E. and Van Niekerk, C.H. 1989a Effect of high levels of dietary molybdenum and sulphate on SA Mutton Merino sheep. I. Mineral status and haematological parameters. *Journal of South African Veterinary Association 19 (3)* : 107-113

Van Niekerk, F.E. and Van Niekerk, C.H. 1989b Effect of high levels of dietary molybdenum and sulphate on SA Mutton Merino sheep. II Certain aspects of the oestrus cycle and pregnancy. *Journal of South African Veterinary Association 19 (3)* : 114-120

Van Niekerk, F.E. and Van Niekerk, C.H. 1989c The influence of experimentally induced copper deficiency on the fertility of rams. I. Semen parameters and peripheral plasma androgen concentration. *Journal of South African Veterinary Association 19 (1)* : 28-31

Van Ryssen, J.B.J. and Stielau, W.J. 1981 Effect of different levels of molybdenum on copper and molybdenum metabolism in sheep fed on high levels of copper. *British Journal of Nutrition 45* : 203-210

Van Ryssen, J.B.J., Botha, W.S. and Stileau, W.J. 1982 Effect of a high copper intake and different levels of molybdenum on the health of sheep. *Journal of the South African Veterinary Association 53 (1)* : 167-170

Van Soest, P.J., Robertson, J.B. and Lewis, B.A. 1991 Methods for dietary fibre, neutral detergent fibre and non starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science 74* : 3583-3597

Vermunt, J.J. and West, D.M. 1994 Predicting copper status in beef cattle using serum copper concentrations. *New Zealand Veterinary Journal 42* : 194-195

Walshe, J.M. 1992 Thiomolybdates in the treatment of Wilsons-Disease. *Archives of Neurology 49 (2)* : 132

Wapnir, D.J. and Stiel, L. 1987 Intestinal absorption of copper: effect of sodium. *Proceedings of the Society of Experimental Biology and Medicine 185* : 277-282

Ward, G.M. 1978 Molybdenum toxicity and hypocuprosis in ruminants: a review. *Journal of Animal Science 46* : 1078-1085

Ward, J.D., Spears, J.W. and Kegley, E.B. 1993 Effect of copper level and source (copper lysine vs. copper sulfate) on copper status, performance and immune response in growing steers fed diets with or without supplemental molybdenum and sulfur. *Journal of Animal Science 71 (10)* : 2748-2755

Ward, J.D., Spears, J.W. and Gengelbach, G.P. 1995 Differences in copper status and copper metabolism among Angus, Simmental and Charolais cattle. *Journal of Animal Science 73* : 571-577

Ward, J.D. Gengelbach, G.P. and Spears, J.W. 1997 The effects of copper deficiency with or without high dietary iron or molybdenum on immune function of cattle. *Journal of Animal Science 75* : 1400-1408

Weber, K.M., Boston, R.C. and Leaver, D.D. 1980 A kinetic model of copper metabolism in sheep. *Australian Journal of Agricultural Research 31* : 773-790

Weber, K.M., Boston, R.C. and Leaver, D.D. 1983 The effect of molybdenum and sulfur on the kinetics of copper metabolism in sheep. *Australian Journal of Agricultural Research 34* : 295-306

Wentink, G.H., Smolders, G., Boxem, Tj., Wensing, T., Müller, K.E. and Van den Top, A.M. 1999 Lack of clinical abnormalities in dairy heifers with low blood and liver copper levels. *Veterinary Record 145 (9)* : 258-259

Wessling-Resnick, M. 1999 Biochemistry of iron uptake. *Critical Reviews in Biochemistry and Molecular Biology 34 (5)* : 285-314

Wiener, G. and Field, A.C. 1971 The concentration of minerals in the blood of genetically diverse groups of sheep. V. Concentrations of copper, calcium, phosphorus, magnesium, potassium and sodium in the blood of lambs and ewes. *Journal of Agricultural Science, Cambridge 76* : 513-520

Wiener, G. and Woolliams, J.A. 1983 Genetic variation in trace element metabolism. In: Suttle, N.F., Gunn, R.G., Allen, W.M., Linklater, K.A. and Wiener, G. (eds) *Trace Elements in Animal Production and Veterinary Practice.* Occasional Publication No. 7- *British Society of Animal Production, Edinburgh, UK*

Wiener, G., Suttle, N.F., Field, A.C., Herbert, J.G. and Woolliams, J.A. 1978 Breed differences in copper metabolism in sheep. *Journal of Agricultural Science, Cambridge 91* : 433-441

Wiener, G., Woolliams, J.A., Suttle, N.F. and Jones, D. 1985 Genetic selection for Cu status in the sheep and its consequences for performance. In: Mills, C.F., Bremner, I. and Chesters, J.K. (eds) *Trace Elements in Man and Animals TEMA-5.* Commonwealth Agricultural Bureaux, Slough.

Wilson, S.A.K. 1912 Progressive lenticular degeneration: a familial nervous disease associated with cirrhosis of the liver. *Brain 34* : 295-509

Wise, T. and Ferrell, C. 1984 Effects of immunisation of heifers against estradiol on growth, reproductive traits and carcass characteristics. *Proceedings of the Society for Experimental Biology and Medicine 176* : 243-248

Wolfensohn, S. and Lloyd, M. 1998 *Handbook of Laboratory Animal Management and Welfare.* 2nd Edition. Blackwell Science Ltd., Oxford, UK

Woodbury, M.R., Feist, M.S., Clark, E.G. and Haigh, J.C. 1999 Osteochondrosis and epiphyseal bone abnormalities associated with copper deficiency in bison calves. *Canadian Veterinary Journal 40* : 878-880

Woolliams, J.A., Suttle, N.F., Wiener, G., Field, A.C. and Woolliams, C. 1982 The effect of breed of sire on the accumulation of copper in lambs, with particular reference to copper toxicity. *Animal Production 35* : 299-307

Woolliams, J.A., Wiener, G., Anderson, P.H. and McMurray, C.H. 1983 Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. *Research in Veterinary Science 34* : 253-256

Woolliams, C., Suttle, N.F., Woolliams, J.A., Jones, D.G. and Wiener, G. 1986a Studies on lambs from lines genetically selected for low and high copper status − Differences in mortality. *Animal Production 43* : 293-301

Woolliams, C., Suttle, N.F., Woolliams, J.A., Jones, D.G. and Wiener, G. 1986b Studies on lambs from lines genetically selected for low and high copper status − incidence of hypocuprosis on improved hill pasture. *Animal Production 43* : 303-317

Wooten, L., Shulze, R.A., Lancey, R.W., Lietzow, M. and Linder, M.C. 1996 Ceruloplasmin is found in milk and amniotic fluid and may have a nutritional role. *Nutritional Biochemistry 7* : 632-639

Xin, Z., Waterman, D.F., Hemken, R.W. and Harmon, R.J. 1991 Effects of copper status on neutrophil function, superoxide dismutase, and copper distribution in steers. *Journal of Dairy Science 74* : 3078-3085

Xin, Z., Silvia, W.J., Waterman, D.F., Hemken, R.W. and Tucker, W.B. 1993 Effect of copper status on luteinizing hormone secretion in dairy steers. *Journal of Dairy Science 76* : 437-444

Zhou, B and Gitschier, J. 1997 hCTR1: a human gene for copper uptake identified by complementation in yeast. *Proceedings of the National Academy of Sciences 94* : 7481-7486

Appendix 1:

Table: 7.2   Blood reference ranges for ovine samples

Sample	Units	Mean	Range
Plasma Copper (Pl-Cu)	µmol/l	15	9.4 - 23
Ceruloplasmin (CP)	mg/100ml	20-25	12-24
Superoxide Dismutase (SOD)	U/g Hb	1350	1102 – 1601
Amine Oxidase (AMOX)	U/l	85	60 - 120
CP:Pl-Cu ratio		1.7	1.0 – 2.5
Liver Copper	mg kg^{-1} DM	300	100-500
Red Blood Cells RBC	X 10^{12}/l	8	5 – 10
Haematocrit (%)	%	0.30	0.24 – 0.40
Haemoglobin (Hb)	g /dl	11	8 – 16
Mean Cell Volume (MCV)	fl	33	23 – 48
Mean Cell Haematocrit (MCH)	pg	10	8 – 12
Mean Cell Haemoglobin Conc. (MCHC)	g / dl	33	31 – 38
White Blood Cells (total) (WBC)	x 10^{9}/l	8.0	4.0 – 10.0
Platelets	x 10^{9}/l	400	250 - 750

Tris-buffered saline (TBS, pH 7.6)

Stock solution (x10):
60.57 g Tris
610 ml distilled water
390 ml HCl 1 N

Phosphate buffered saline (0.1 M PBS, pH 7.2)

Stock solution (x10):
80 g Sodium Chloride
2 g Potassium Chloride
11.5 g disodium hydrogen phosphate
2 g Potassium dihydrogen phosphate

Dissolve in 1000 ml of distilled water

Imidazole/HCl buffer (0.1M, pH 7.1)

6.81 g Imidazol in 1000 ml distilled water
Add 500 ml of 0.1M HCl until pH 7.2 is maintained

Diaminobenzidintetrahydrochloride Solution (DAB)

Dilute 100 mg DAB in 200 ml of 0.1M Imidazole/HCl buffer (0.1M, pH 7.1)
Mix thoroughly using magnetic stirrer
Filter and add 70μl Hydrogen Peroxide directly before use

Papanicolaou's solution

Mix Papanicolaou's solution and distilled water (1:20) and filter

244