THESIS

EFFECTS OF COPPER SOURCE AND CONCENTRATION ON COPPER STATUS AND IMMUNE FUNCTION IN GROWING AND FINISHING STEERS

Submitted by

Kristy L. Dorton

Department of Animal Science

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY KRISTY L. DORTON ENTITLED EFFECTS OF COPPER SOURCE AND CONCENTRATION ON COPPER STATUS AND IMMUNE FUNCTION OF GROWING AND FINISHING STEERS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Adviser

Department Head/Director

ABSTRACT OF THESIS

EFFECTS OF COPPER SOURCE AND CONCENTRATION ON COPPER STATUS AND IMMUNE FUNCTION OF GROWING AND FINISHING STEERS

Forty-eight, individually fed, purebred Angus steers (approximately 7 months of age, 218 + 9.1 kg) were used to determine the effects of copper (Cu) source and concentration on performance, Cu status, and immune function. Steers were stratified by weight and initial liver Cu concentration and randomly assigned to one of five treatments. Treatments consisted of: 1) control (no supplemental Cu), 2) 10 mg Cu/kg DM from Cu sulfate ($CuSO_4$), 3) 10 mg Cu/kg DM from a Cu-amino acid complex (Availa Cu), 4) 20 mg Cu/kg DM from $CuSO_4$, and 5) 20 mg Cu/kg DM from Availa Cu. Steers were housed in individual pens and fed an alfalfa-corn based growing diet for 56 d then were fed a high concentrate diet for 144 d. On d 56 of the growing phase and d 112 of the finishing phase, liver Cu concentrations were higher ($P < 0.01$) in Cu supplemented steers. Steers receiving 20 mg Cu/kg DM had higher ($P < 0.01$) liver Cu concentrations than steers receiving 10 mg Cu/kg DM. On d 112 of the finishing phase, steers receiving 20 mg Cu/kg DM from Availa Cu had higher ($P < 0.01$) liver copper concentrations than steers supplemented 20 mg Cu/kg DM from $CuSO_4$. In the growing phase, steers supplemented with 20 mg Cu/kg DM had greater ($P < 0.04$) plasma Cu concentrations than steers supplemented with 10 mg Cu/kg DM. In the growing and finishing phases, plasma Cu concentrations were higher ($P < 0.03$) in steers supplemented with 20 mg Cu/kg DM from Availa Cu than steers receiving 20 mg Cu/kg DM from $CuSO_4$. Cell

mediated immune response to phytohemagglutinin was higher ($P < 0.01$) in steers supplemented with 20 mg Cu/kg DM than 10 mg Cu/kg DM treatments. Total immunoglobulin (Ig) and immunoglobulin G (IgG) concentrations specific to pig red blood cells (PRBC) were greater ($P < 0.01$) in steers supplemented with 10 mg Cu/kg DM from $CuSO_4$ than 10 mg Cu/kg DM from Availa Cu. During the finishing phase, IgG concentrations specific to PRBC were higher ($P < 0.03$) and antibody titers specific to ovalbumin (OVA) were lower ($P < 0.05$) in control steers relative to Cu supplemented steers. Steers receiving 20 mg Cu/kg DM had higher antibody titers specific to OVA ($P < 0.02$) than 10 mg Cu/kg DM steers. Steers receiving 20 mg Cu/kg DM from Availa Cu had higher ($P < 0.01$) antibody titers specific to OVA than steers supplemented with 20 mg Cu/kg DM from $CuSO_4$. These results indicate that Cu source and concentration may have an effect on the immune system. Furthermore, it appears that the immune response to an antigen varies depending on the type of antigen administered as well as the concentration and source of Cu supplemented.

Kristy L. Dorton
Animal Science Department
Colorado State University
Fort Collins, CO 80523
Summer 2002

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Chapter 1

INTRODUCTION

Several biochemical and physiological changes have been reported in copper (Cu) deficient animals including reduced appetite, reduced growth, as well as impairment of several metabolic enzymes. Copper deficiency has also been associated with reduced immune function and increased susceptibility to disease (Stable and Spears, 1990). Many aspects of the immune system are impaired by Cu deficiency including cell mediated and humoral immunity (Prohaska and Failla, 1993), superoxide dismutase activity (Jones and Suttle, 1981; Stabel and Spears, 1990; Gengelbach et al., 1997), T cell proliferation, cytokine production, and antibody production (Stabel and Spears, 1990). Furthermore, genetic and environmental factors have been reported to impact an animals' ability to respond immunologically to an antigen (Nguyen, 1984). Therefore, proper management of feedlot cattle can help to reduce the impact of environmental factors by enhancing the animal's immune system via vaccination and proper nutrition. In most basal feedlot diets, Cu concentrations and availability are typically low; therefore Cu is commonly added to feedlot rations. Supplemental forms of Cu include both inorganic and organic sources. Recently, there has been considerable interest in the use of organic trace minerals in cattle diets because of research indicating improved bioavailability as compared to inorganic forms (Henry et al., 1992; Nockels et al., 1993; Rojas et al., 1995).

Therefore, the objective of this study was to determine the effects of Cu concentration and source on Cu status and immune function in growing and finishing feedlot steers.

Chapter 2

LITERTURE REVIEW

Chemical Properties of Copper

Copper has a dull lustrous reddish brown color. It is the 29^{th} element and is in group IB of the periodic table. Its symbol is Cu. The relative atomic mass of Cu is 63.546 (Lide and Frederikse, 1993; McDowell, 1992). Copper has a melting point of 1083.4°C, a boiling point of 2567°C, and is insoluble in water (Lide and Frederikse, 1993). Copper is malleable, a good thermal conductor, and is an excellent electrical conductor (Dameron and Howe, 1998). Copper has four oxidation states: metallic Cu (Cu^{o}), cuprous ion (Cu(I) or Cu^{+}), cupric ion (Cu(II) or Cu^{2+}), and trivalent ion (Cu^{3+}). Copper can form organometallic compounds and is found in a variety of mineral salts and organic compounds in the elemental or metallic forms (Dameron and Howe, 1998). The metallic form is stable in dry air at low temperatures, but in moist air a slow reaction occurs. During this reaction hydrocarbonate or hydrosulfate is formed which causes a greenish gray amorphous film to develop that protects the metal underneath from further attack by the air (Dameron and Howe, 1998). Cotton and Wilkinson (1989) stated that Cu can be dissolved in highly acidic solutions and in certain basic solutions.

The chemical properties of Cu depend on the electronic or oxidative state that Cu resides. The ease at which Cu changes oxidation state gives Cu redox properties that can be both beneficial and deleterious for biological systems (Dameron and Howe, 1998).

Copper(I) and Cu(II) are the most common oxidation states with Cu(I) being quickly oxidized by oxidizing reagents and Cu(II) being the most important oxidation state in a natural, aqueous environment (Dameron and Howe, 1998). Chemical behaviors determine the metabolism of Cu because these chemical behaviors determine how Cu will interact with other minerals and compounds during absorption, transport, and distribution of Cu to body tissues (Dameron and Howe, 1998).

Metabolism of Copper: Digestion, Absorption, Transport, Storage, Excretion

Weak Cu complexes are dissociated at a low pH (Allen and Solomons, 1984; Dameron and Howe, 1998). This occurs when food enters the stomach (the abomassum in ruminants). Once dissociated in the stomach, Cu may bind to amino acids or oligopeptides (Allen and Solomons, 1984). Gollan and Dellor (1973) stated that it is likely that low molecular weight substances, such as certain amino acids in gastrointestinal (GI) secretions will bind to Cu and maintain Cu in solution in the upper small intestine. According to Marceau et al. (1970), Cu may be bound to certain amino acid complexes before it is absorbed into the gut.

Copper absorption is regulated by the metabolic need of an animal (McDowell, 1992). Intestinal absorption is influenced by the chemical form that Cu is in and by interactions that Cu has with other components of the diet (McDowell, 1992). McDowell (1992) stated that Cu is poorly absorbed in most species. Ruminants absorb only one to three percent of Cu, while non-ruminants are able to absorb five to ten percent of Cu (McDowell, 1992). Absorption occurs primarily in the upper portion of the small intestine (Allen and Solomon, 1984; Linder, 1991; McDowell, 1992; Linder et al., 1999).

Absorption can occur in other parts of the GI tract as well, but the amount absorbed is much lower than that absorbed in the upper small intestine (Dameron and Howe, 1998).

Copper is absorbed across the intestinal mucosa in a two step process (Crampton et al., 1965). First, Cu is transported across the mucosal membrane. Then, it is transported across the cell. Copper can be absorbed through one of two mechanisms: saturable (active transport) or unsaturable (simple diffusion) (Allen and Solomons, 1984; Bronnerd and Yost, 1985; Dameron and Howe, 1998). On the basolateral side of the intestinal cell, transport out of the cell seems to be energy dependent and may limit the rate of Cu transfer to albumin (Allen and Solomons, 1984; Dameron and Howe, 1998). If high levels of Cu are consumed or if body stores are adequate, other energy dependent mechanisms are activated to bring Cu into the cell and Cu will compete with other metals for places to enter the cells (Linder, 1991).

Copper absorption may be regulated by Cu status of the body. Copper absorption is increased when lower levels of Cu are present in the diet (Strickland et al., 1972; Giltin et al., 1960). Copper transfer into the blood may be regulated by cystol protein that enhances or blocks the transfer of Cu into the blood (Allen and Solomons, 1984). Copper absorption may also be regulated or more likely restricted by metallothionin (Allen and Solomons, 1984). Metallothionin has a high affinity for Cu and will bind Cu very readily and keep it from being absorbed.

Once Cu is absorbed across the small intestine into the blood stream, Cu becomes attached to albumin (Alb), amino acids (such as histidine (His)), or transcuprien (Lau et al., 1974; Schmitt et al., 1983; McArdle et al., 1999). Copper in the blood is primarily bound to albumin (Peters and Hawn, 1967), which has specific and non-specific binding

sites for Cu (McArdle et al., 1999; Linder et al., 1999). Small amounts are bound in plasma to amino acids (Anon, 1981). Transcuprein also binds Cu. Linder et al. (1999) stated that transcuprein has an N-terminal amino acid that is like the high affinity binding site on albumin. Transcuprein has a higher affinity for Cu than albumin and readily exchanges Cu(II) with albumin (Linder et al., 1999).

Once bound to these compounds, Cu is carried through the blood stream and is taken up primarily by the liver and kidney (Linder et al., 1999). The liver is very important in the metabolism of Cu (McArdle et al., 1999) and is the primary organ for Cu storage and distribution (Dameron and Howe, 1998). Uptake across the hepatic membrane is believed to be carrier mediated (Ettinger et al., 1986; McArdle et al., 1999) and is not dependent on cell energy or coupled to the sodium (Na) gradient (McArdle et al., 1999). Hepatocytes remove Cu from the high affinity sites on albumin, however, copper can be transported across the hepatic membranes from any Cu transporting complex (McArdle et al., 1990). The transporter that brings Cu into the hepatic cell membrane is a dimer attached to two disulfide bridges (McArdle et al., 1999). One of the bridges is on the outside of the hepatic membrane, while the second is on the inside of the membrane (McArdle et al., 1999). McArdle et al. (1999) stated that little is truly known about how Cu is transported across the hepatic cell membrane, however, it has been reported that Cu(II) if first reduced to Cu(I) by NADH oxidase and then transported into the hepatocyte. Copper(I) then enters the cell through the disulfide bridge and is reoxidized. It is not known whether Cu(I) is reoxidized to Cu(II) before or after Cu enters the Golgi apparatus in the hepatic cell. Copper is transported across the Golgi apparatus by ATP7B, which is energy dependent (McArdle et al., 1999).

Within the liver, there are various pools that Cu is divided into. These are the transit, storage, and excretion pools (Owen, 1980). Copper within the transit pool is used or incorporated into ceruloplasm and other Cu dependent enzymes such as Cu-Zn superoxide dismutase (SOD). Ceruloplasm binds six Cu atoms tightly and one Cu atom loosely near the surface of the molecule (Zaitseva et al., 1996). Sixty to ninety percent of Cu in plasma is bound to ceruloplasm with the rest being bound to albumin, transcuprein, and ligands (McArdle et al., 1999). Ceruloplasm and bound Cu is transported to specific tissues throughout the body (Linder 1991; Weiss et al., 1985, McDowell, 1992) utilizing specific receptors and transporters for these molecules (Linder et al., 1999).

Copper that is placed into the storage pool is stored in the liver until it is needed. The liver is the major storage organ for Cu, and Cu is stored in the form of mitochondrial cuprein (McDowell, 1992). The liver has the capacity to store approximately 20% of the body's Cu supply. Muscle tissue (40%), brain (20%), connective tissue (8%), blood (8%) and kidneys (8%) can store and utilize Cu (Dameron and Howe, 1998), however the liver is the true storage organ for the body. Copper in the liver can be mobilized and shipped to extrahepatic tissues when the body is in a negative Cu balance (Dameron and Howe, 1998), while tissues and blood utilize Cu and do not supply other tissues with appreciable amounts of Cu. The amount of Cu stored is dependent on Cu intake and Cu status of an animal (McDowell, 1992). Metallothionin may be essential or may play a role in the storage of Cu (McArdle et al., 1999). Metallothionin (a class of low molecular weight binding proteins) can bind up to twelve Cu(I) atoms. This aids in Cu storage as well as protecting the body from Cu toxicities (Bremner, 1987). Copper can be moved from the storage pool to the transit pool if necessary when the body is in a negative Cu balance.

Copper that is placed into the excretion pool is excreted out of the body. Copper is excreted primary in feces (McDowell, 1992) and the amount is dependent on Cu absorbed from the diet (Underwood, 1977). Excretion occurs mostly through bile (Committee on Animal Nutrition et al., 1980; Allen and Solomons, 1984; McDowell, 1992; Dameron and Howe, 1998). Eighty percent of Cu excreted from the body is excreted through bile (Winge and Mehra, 1990). Copper can complex with bile salts in the canaliculi (McArdle et al., 1999) or through bilary excretion of glutathione (Freedman et al., 1989). Some of the Cu that is secreted back into the GI tract is unavailable for reabsorption (Linder et al., 1999). Copper can also be excreted in urine (Underwood, 1977; Harris, 1991; Linder et al., 1999), milk, sloughed off intestinal cells, and through perspiration, but the amount excreted via these mechanisms is small (Underwood, 1977).

Physiological functions of Cu

Underwood (1981) stated that there are three basic functions of minerals: 1) they can be structural components of body organs and tissues, 2) minerals can be constituents of body fluids and tissues as electrolytes concerned with acid-base balance, membrane permeability, and tissue irritability, and 3) they can also be catalysts in enzymes and hormone systems.

Copper is an essential trace mineral that is required by the body in order for the body to function normally. Copper plays a role in activating a number of enzymes (Committee on Animal Nutrition et al., 1980; Dameron and Howe, 1998). Copper is also incorporated into a vast amount of enzymes and structural proteins. Copper is an essential component of these metalloenzymes (McDowell, 1992; Dameron and Howe, 1998; Baker and Ammerman, 1995). It is second only to zinc in the number of enzymes

that it functions within (Committee on Animal Nutrition et al., 1980). Cytochrome C oxidase, lysyl oxidase, superoxide dismutase, dopamine β hydroxylase, and tyrosine are the major Cu dependent enzymes that have been extensively researched.

Because of the vast array of enzymes that Cu activates and is incorporated into, Cu has many functions throughout the body. Copper plays a role in iron (Fe) metabolism. Copper is important for Fe absorption and mobilization. Ceruloplasm, which contains Cu, is necessary for oxidation of Fe (McDowell, 1992). Ceruloplasm catalyzes the conversion of Fe^{2+} (ferrous form) to Fe^{3+} (ferric form), which is necessary for Fe to be transported as transferrin throughout the body (McDowell, 1992). Copper is necessary for hemoglobin synthesis, serving as a catalyst before the body can utilize Fe for hemoglobin formation (McDowell, 1992), and in erythropoiesis (Committee on Animal Nutrition et al., 1980; Baker and Ammerman, 1995; Dameron and Howe, 1998).

Copper has been shown to be essential in cellular respiration. Copper is a component of cytrochrome C oxidase (Allen and Solomons, 1984; Dameron and Howe, 1998; Linder et al., 1999). Cytochrome C oxidase is a terminal oxidase in cellular respiration chain (McDowell, 1992; Baker and Ammerman, 1995). It catalyzes the reduction of oxygen (O_2) to water (H_2O; McDowell, 1992).

Copper is an important component in the cross-linking of connective tissues. Lysyl oxidase is a Cu containing enzyme that is a key component in the formation of cross linkages in collagen and elastin (Allen and Solomons, 1984; McDowell, 1992; Linder et al., 1999). Collagen and elastin are key components in connective tissue, blood vessels, and gives strength and stability to bones (Committee on Animal Nutrition et al.,

1980). Lysyl oxidase is also important in reproduction, bone development, and growth (Committee on Animal Nutrition et al., 1980; Dameron and Howe, 1998)

Pigmentation and keratinization of hair and wool are dependent on Cu. Copper is a component of tyrosinase, which is the enzyme that catalyzes the conversion of tyrosine to melanin (Committee on Animal Nutrition et al., 1980; Allen and Solomons, 1984; McDowell, 1992). Melanin is responsible for the pigmentation of hair and wool. Copper is also required for the formation and/or is incorporated into disulfide groups in keratin synthesis (McDowell, 1992). These disulfide groups provide cross linkages or bonding of keratin.

The central nervous system is dependent on Cu for proper function. Cytochrome C oxidase is the key enzyme in this system because this enzyme is essential in the myelination of nerve fibers. Cytochrome C oxidase is important in the production of phospholipids in the liver, which makes up a large portion of myelin (Zimmerman et al., 1976; McDowell, 1992).

Copper also provides protection against oxidants. Impaired Fe metabolism can promote free radical generation (Committee on Animal Nutrition, 1980; Dameron and Howe, 1989). Copper causes the activation of a Fe containing heme enzyme catalase (Committee on Animal Nutrition et al., 1980). This catalase protects tissues from hydrogen peroxide (H_2O_2) and hydroperoxide damage (Golden and Ramdath, 1987). Ceruloplasm is believed to aid in antioxidant defense. Saenko et al. (1994) reported that ceruloplasm is believed to scavenge Fe radicals and other free radicals in the body that could cause tissue damage. Copper-zinc superoxide dismutase (Cu-Zn SOD) is another Cu containing enzyme that aids in antioxidant defense (Committee on Animal Nutrition

et al., 1980). This enzyme is responsible for ridding the body of superoxide radicals (O_2^-) that cause tissue degradation. Copper-Zn SOD will be discussed in more depth below.

There are many more functions that Cu has in the body. Copper plays a role in reproduction and lipid metabolism. It also is important for proper function of the immune system. Without Cu, the enzymes listed above would not be activated or produced and the systems the enzymes are incorporated into would not be able to function normally.

Immune system and Cu relationship

Copper has been shown to be essential for normal function of the immune system (Committee on Animal Nutrition et al., 1980). Copper deficiency can cause impaired immunity (Stabel and Spears, 1990). Immunity is a reaction by the body to a foreign substance, such as microbes and/or a virus (Abbas et al., 1991). Immunity is divided into two categories: innate and acquired immunity. Innate immunity is the basic resistance to disease (Engle, 2001). It is the physical and chemical barriers to disease (Abbas et al., 1991). Kuby (1994) stated that innate immunity is composed of four barriers: 1) anatomic (skin), 2) physiologic (temperature or pH), 3) phagocytic (ingestion of macromolecules by macrophages and neutrophils), and 4) inflammatory (vasodilation and capillary permeability). Acquired immunity is stimulated by exposure to an antigen through vaccination or natural exposure (Abbas et al., 1991; Kuby, 1994). Acquired immunity can be divided into two categories: humoral and cell-mediated immunity. Humoral immune response is mediated by B-cells that respond to antigens to become antibody producing cells and memory cells (Galyean et al., 1999; Engle, 2001). B-cells and antibodies provide defense against extracellular infections (Galyean et al., 1999) via

antibodies binding to the antigen outside of the cell, neutralizing them and causing the antigens' elimination by other immune cells (Engle, 2001). Cell-mediated immune response is mediated by T-cells and their associated cytokines (Galyean et al., 1999). Galyean et al. (1999) stated that cell-mediated immunity provides a defense against intracellular pathogens and tumor cells.

According to Kuby (1994), an immune response goes as follows. Immune cells, such as macrophages, roam the body and engulf (sample) cells. They sample both self (body) and non-self (foreign bodies) cells because macrophage are unable to tell the difference between these types of cells. The macrophage will engulf body cells/antigen and break the cells apart. Pieces of the body cell/antigen are presented on the outside of the macrophage. T-cells will attach to the presented piece of the body cell/antigen. T-cells have the ability to distinguish between self and non-self cells. If the presented cell is a self-cell, the T-cell will detach and move on to the next macrophage. If the presented cell is an antigen, the T-cell will bind strongly to the antigen and the macrophage and cause a cascade of events to occur. Cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), will be released from the T-cell and macrophage upon recognition of a foreign pathogen presented by a macrophage. Cytokines activate T-cells and B-cells to proliferate or divide into more T-cells and B-cells and also into memory T-cells and B-cells. The activated T-cells bind to more antigen on macrophage and cause more cytokines to be released. B-cells will produce and release antibodies that will bind to the free antigens in the infected area. When bound, the antibodies mark the antigen for destruction by the complement system, which splits the antigen into pieces. Macrophage will bind to the antibodies on the antigen, which allows the macrophage to engulf the

antigen more efficiently. Cytokines also "recruit" other macrophage and killer T-cells, whose purpose is to destroy infected body cells that cannot be saved once infected with the antigen. This proliferation and recruitment of more immune cells increases the immune response. When the antigen is eradicated from the body, suppressor T-cells will come into the previously infected area and decrease the immune response. If the same antigen enters the body again, the memory T-cells and B-cells will be activated to rid the body of the antigen. Memory T-cells and B-cells will cause the secondary immune response to be faster at ridding the body of the antigen because the immune cells are already produced and are specific for this antigen (Kuby, 1994).

Research on Cu effects on the immune system has shown inconsistent results. Results seem to vary between species, breed, environment the research is being conducted in, and area of the immune system being studied. Discussed below are a few aspects of the immune system and its relationship with Cu.

Antibodies (Immunoglobulin G (IgG) and Immunoglobulin M (IgM)): Antibodies (immunoglobulins) are produced by B-cells when B-cells are activated by cytokines. Kuby (1994) stated that antibodies, once secreted, will circulate in the blood and search out and neutralize or eliminate antigens. This is part of the humoral immune response. There are various isotypes of antibodies that have different functions. Two of these isotypes are IgG and IgM. Immunoglobulin G is the most abundant isotype in serum (Kuby, 1994). Immunoglobulin M only accounts for 5-10% of total serum immunoglobulin and is the first antibody to be released during an initial immune response (Kuby, 1994).

There have been minimal studies that have examined Cu's effects on total IgM and IgG as an indicator of overall immune response. Stabel et al. (1993) conducted a study using fourteen Holstein steers. Steers received a milk replacer diet containing a mineral mix then were fed a starter diet containing cottonseed hulls, cornstarch, and urea. The basal diet contained 1.5 mg of Cu/kg DM. Steers were then placed on one of two treatments: 1) no supplemental Cu or 2) 10 mg of Cu/kg DM from $CuSO_4$. They reported that total IgM and IgG were not affected by Cu status in Cu-adequate and Cu-deficient Holstein calves (Stable et al., 1993).

Ward et al. (1997) also examined IgM and IgG in cattle. They used forty, 2-year-old Angus, Simmental, and Charolais heifers that were depleted of Cu for 17 d using 20 mg Mo/kg DM. The basal diet was a corn silage based diets that met or exceeded the NRC recommendations for pregnant heifers, with the exception of Cu. The basal diets contained the following Cu concentrations: control and gestation diets contained 4 mg Cu/kg DM and the lactation diet contained 4.5 mg Cu/kg DM. Calves were bled 7 d after birth. They found that treatment had no effect on total IgG concentration in the serum of calves.

Cell-mediated immune response to Phytohemagglutinin (PHA): Kuby (1994) stated that cell-mediated immunity is the T-cell dependent branch of the immune system. Cell-mediated immunity is responsible for ridding the body of intracellular pathogens, virus-infected cells, and tumor cells. Phytohemagglutinin is often used to determine the effects of various minerals on cell-mediated immunity. Phytohemagglutinin is a kidney bean lectin (protein) that has mitogenic activity, therefore, PHA is able to induce cell division in a high percentage of T-cells with varying antigen specificity (Kuby, 1994).

When injecting PHA or any other antigen into the body, the cell-mediated branch of the immune system responds to destroy this foreign substance. Results tend to vary between studies. Ward et al. (1993) conducted a study using 126 Angus and Angus x Hereford crossbred steers. The steers were fed a control diet containing no supplemental minerals, or they were supplemented with 5 mg Mo/kg DM plus 0.2% S from MoS, 5 mg Cu/kg DM from CuSO4, 5 mg Cu/kg DM from $CuSO_4$ plus 5 mg Mo and 0.2% S from MoS, 5 mg Cu/kg DM from CuLys, or 5 mg Cu/kg DM from CuLys plus 5 mg Mo and 0.2% S from MoS. The basal diet contained 6.2 mg Cu/kg DM. Cell-mediated immunity was measured on d 7 and d 77 by injecting 150 ug of PHA intradermally and skinfold thickness was measured prior to injection and 4, 8, 12, 24, and 48 h post-injection. On d 7, there was not a treatment effect on cell-mediated immunity. On d 77, Cu had no effect on swelling response, while Mo and S supplemented steers had a decrease in reactivity. Ward et al. (1993) reported that cell-mediated immunity might be more susceptible to Mo and S supplementation. In 1997, Ward et al. conducted a study using forty 2-year old pregnant Angus, Simmental, and Charolais heifers in their last trimester of pregnancy. They supplemented the heifers with 600 mg Fe/kg DM from $FeCO_3$, 5 mg Mo/kg DM from $NaMoO_4$, 10 mg Cu/kg DM from $CuSO_4$, or gave no supplemental minerals. The supplements were added to a basal diet containing 4 mg Cu/kg DM (depletion and gestation diets) and 4.5 mg Cu/kg DM (lactation diet). The lactation diet was fed once the heifers calved. The calves of the heifers were injected intradermally with 150 ug of PHA 286 d into the study and skinfold thickness was measured prior to injection and at 4, 8, 12, 24, and 48 h post injection. The results showed that Cu supplemented calves had lower swelling responses to PHA injections than all other treatment groups at 4, 8, 12,

and 24 hours post injection (Ward et al., 1997). Ward et al. (1997) conducted another study where they fed a control diet deficient in Cu or supplemented 10 mg Cu/kg from $CuSO_4$ or 5 mg Mo/kg DM from $NaMoO_4$ to Holstein bull calves to a basal diet containing 1.1 mg Cu/kg DM. The calves were injected with PHA intradermally and skinfold measurements were measured prior to injections and 2, 4, 6, 8, 12, and 24 h post injections. Cu supplemented steers had a greater ($P < 0.06$) immune response to PHA than did control steers at 12h and 24 h post injection (Ward et al., 1997). The results varied between the two studies. Ward et al. (1997) stated that the differences may have been caused by breed differences and diet differences but not age because both sets of calves were all injected when they were approximately 180 d old.

Superoxide Dismutase (SOD): When phagocytic cells ingest and breakdown antigens or other cells, superoxide radicals (anions) are produced (Stabel and Spears, 1990). Superoxide anions (O_2^-) can cause oxidative damage to cellular membranes (Stabel and Spears, 1990). These anions break down tissues in the body. A Cu containing enzyme, Cu-Zn SOD, is important in inhibiting the harmful effects of O_2^-. It is part of the following reaction:

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

Then,

$$H_2O_2 + 2GSH \xrightarrow{GSH\text{-}PX} GSSG + 2H_2O$$

Superoxide dismutase converts O_2^- to hydrogen peroxide (H_2O_2) and oxygen (O_2). Glutathione peroxidase (GSH-PX) then reduces H_2O_2 to water (H_2O) decreasing the harmful effects of the O_2^- (McMurray et al., 2000).

Superoxide dismutase contains 2 atoms of Cu and 2 atoms of Zn, and Cu contained within the enzyme is important for catalytic activity (Paytner, 1987). When Cu is sufficient in the diet, SOD activity seems to be unaffected. In a trial by Ward et al. (1993) (diet and treatments mentioned above), blood samples were taken from 72 of 126 Angus and Angus x Hereford steers on d 21, 42, 63, and 84 of the study. The samples were centrifuged and the red blood cells (RBC) were lysed. The lysed RBC were analyzed using a procedure described by Jones and Suttle (1981). Ward et al. (1993) found no difference among treatments in SOD activity when supplementing $CuSO_4$ or CuLys verses no supplementation to steers. Torre et al. (1995) conducted a study using 10 primigravid Holstein heifers that were around 2-years of age. The heifers received no supplemental Cu from 16 to 20 weeks of age. When the heifers reached approximately 84 d prior to parturition, they were randomly assigned to receive the basal diet or the basal diet supplemented with 20 mg Cu/kg DM from $CuSO_4$. The basal diet was composed of alfalfa silage, corn silage, and cottonseed and contained 6 to 7 mg Cu/kg DM. Superoxide dismutase activity was measured from these heifers in lysed whole blood. The results showed that there were no differences in SOD activity between treatments.

In the above studies, RBC SOD was unaffected and Cu deficiency was not present. Ward et al. (1993) stated that they believed that if they had continued their experiment for more that 98 d, SOD activity may have been decreased in steers supplemented with Mo and S because Cu deficiency may have occurred.

It is believed that RBC SOD activity is only affected during a severe, prolonged Cu deficiency (Paytner, 1987). Copper deficiency is reached when liver Cu

concentration are less than 20 mg Cu/kg DM and when plasma Cu concentrations are less than 0.6 mg Cu/L (Mills, 1987). A severe, prolonged deficiency would be when liver and plasma Cu concentrations would be less than these levels for prolonged periods of time. Paynter (1987) stated that SOD in RBC is one of the last enzymes that is affected by Cu deficiency. He stated that RBC SOD has a longer half-life than other enzymes, such as plasma ceruloplasmin, because RBC have a longer half-life, which could explain why RBC SOD is effected when Cu deficiency is more severe (Paytner, 1987). Paytner (1987) also reported that RBC SOD activity is closely related with liver Cu status and with enzyme activities in the liver.

Superoxide dismutase activity differences have been seen in studies where Cu deficiency is observed. Andrewartha and Caple (1980) conducted a studying using 7 nine-month old Corriedale rams. Four rams were supplemented with 20 mg Mo per day from sodium molybdate and 6 g sulfate/d from ammonium sulphate to a commercial sheep diet containing 10 to 12 mg Cu/kg DM and the remaining 3 rams were fed only the commercial sheep diet. Blood samples were taken prior to initial use of the depletion diet and 90 d after feeding the diet. The RBC were lysed and analyzed for SOD activity using a method described by Beauchamp and Fridovich (1971). Rams in this study that were depleted of Cu by adding molybdenum and sulfur to the diet had RBC SOD activity 40% lower than the control rams (Andrewartha and Caple, 1980). In a separate study using deficient lambs that grazed a pasture known to be deficient in Cu, SOD activity increased when lambs were fed the commercial sheep diet containing 10 to 12 mg Cu/kg DM (Andrewartha and Caple, 1980). Jones and Suttle (1981) utilized eight Scottish Blackface ewes that were fed a Cu deficient diet and six Scottish Blackface cross Border

Leicester ewes that were fed a complete diet containing an adequate amount of Cu (controls). Blood samples for SOD activity were taken and the RBC were lysed and analyzed using optical density readings. The sheep had plasma Cu concentrations that were less than 0.5 mg/L, which is considered deficient according to the levels mentioned above (< 0.6 mg/L; Mills, 1987). The deficient sheep had lower SOD activity than control sheep (Jones and Suttle, 1981). In another study by Xin et al. (1991), twelve Holstein steers were supplemented with 20 mg Cu/kg DM from $CuSO_4$ or 10 mg Mo/kg DM from ammonium molybdate to a basal diet containing alfalfa haylage and concentrate for the first 5 months or fescue hay and concentrate for the last 3 months that contained 18 to 30 mg Cu/kg DM. Blood samples were taken prior to being fed the treatments and once a month until the end of the trial. Red blood cells were lysed and analyzed using a modified technique described by Jones and Suttle (1981). Deficient steers had plasma Cu concentrations less than 0.8 mg/L and liver Cu concentrations less than 19 mg/kg, which are considered deficient or almost deficient as mentioned above (< 0.6 mg/L and < 20 mg/kg; Mills, 1987). SOD activity was lower ($P < 0.01$) in Cu deficient steers than Cu supplemented cattle (Xin et al, 1991).

According to above mentioned studies, it appears that an animal needs to be Cu deficient in order to see a decrease in SOD activity. Without this severe deficiency, SOD activity would remain within normal levels.

Primary and Secondary Humoral Immune Response to Pig red blood cells (PRBC) and ovalbumin: As mentioned earlier, the humoral branch of the immune system involves the interaction of B-cells with antigen (Kuby, 1994). It involves the differentiation and proliferation of these B-cells into antibody secreting plasma cells and

memory cells (Kuby, 1994). A primary immune response is when the body is first introduced to an antigen (Kuby, 1994). The body has to produce T-cells, B-cells, antibodies, and other immune cells to respond to this antigen. Memory T-cells and B-cells are also produced and lie dormant until the same antigen enters the body. When the same antigen reenters the body, a secondary immune response is the result where the response to the antigen will be rapid and heightened because memory immune cells are already present in the body to combat the antigen and do not have to be produced (Kuby, 1994). During a primary immune response, IgM is the first immunoglobulin that is produced to combat an antigen and less IgM is needed to neutralize a virus than IgG (Kuby, 1994). During a secondary immune response, IgG is released first (Kuby, 1994). The antibodies produced during a secondary immune response have a higher affinity for the antigen and antibody levels are often 100 to 1000 times higher than in the primary immune response (Kuby, 1994).

Antigens are often injected into animals in order to elicit an immune response to observe how humoral immunity is affected by mineral, protein, or substance a study is testing. One of these antigens is pig red blood cells (PRBC). If injected into species other than swine, the PRBC are a foreign substance. The body will set the immune system into action to rid the body of this foreign substance. Pig red blood cells are often diluted in phosphate buffered saline (PBS) in varying concentrations (examples: 20% and 25% PRBC). The solution is injected into the neck muscle of the animal and blood samples are usually taken prior to injection and 7, 14, and 21d post-injection. The serum is then analyzed for antibody titers.

Results from studies where PRBC were injected have shown varying outcomes. In 1997, Ward et al. conducted a study using calves whose mothers (40 2-year old Angus, Simmental, and Charolais heifers) were supplemented with 600 mg Fe/kg DM from $FeCO_3$, 5 mg Mo/kg DM from $NaMoO_4$, 10 mg Cu/kg DM from $CuSO_4$, or gave no supplemental minerals to a basal diet containing 4 mg Cu/kg DM during gestation and 4.5 mg Cu/kg DM during lactation. The calves where injected with 5 mL of a 20% PRBC solution on d 169 of the study when the calves were approximately 70 d old (primary immune response) and then again on d 246 when the calves were approximately 147 d old (secondary immune response). Blood samples were taken prior to injection and 7, 14, 21, and 28 d post injection. The serum was then analyzed for total antibody titers specific for PRBC using a procedure described by Droke et al. (1993). There was not a treatment effect after primary injection; however, calves supplemented with Cu had less antibody titers than control steers 14 d ($P < 0.07$) and 21 d ($P < 0.09$) post-secondary injection (Ward et al., 1997). In another study, Ward and Spears (1999) supplemented 42 Angus bull calves with 0 or 5 mg Cu/kg DM from $CuSO_4$, or 0 or 5 mg Mo/kg DM from Na_2MoO_4 to a diet containing 5.2 mg Cu/kg DM. Calves were injected with 10 mL of 25% PRBC solution on d 133 (primary immune response). Blood samples were collected prior to injection and 7, 14, 21,and 28 d post injection. Calves that were supplemented with Cu had less ($P < 0.07$) antibody titers to PBRC than control steers (Ward and Spears, 1999).

Another antigen that is injected into animals to elicit a humoral immune response is ovalbumin (OVA). Ovalbumin is found on the inner part of an egg shell. Ovalbumin is often mixed into a solution with PBS and Freund's incomplete adjuvant (FIA).

Freund's incomplete adjuvant boosts the immune response when an antigen has low immunogenicity, prolongs antigen persistence from days to weeks, and induces granuloma formation that may be responsible for facilitating antigen processing and presentation and T helper cell activation (Kuby 1994). In short, it causes a heightened immune response.

Studies where OVA were injected have also shown varying results as well. In the study mentioned above by Ward and Spears (1999), four milliliters of solution containing 4 mg of OVA in PBS and FIA was injected subcutaneously into the calves on d 133. Blood samples were take prior to injection and 7, 14, 21, and 28 d post injection. Serum was analyzed for antibody titer concentrations specific to OVA using a modified procedure of Droke and Loerch (1989). Ward and Spears (1999) reported that immune response to OVA was greater in Angus bull calves supplemented with Cu than in control steers 7, 14, 21, and 28 d post injection ($P < 0.06$). Ward et al. (1993) showed opposite results when conducting a study using 126 Angus and Angus x Hereford crossbred steers. The steers were assigned to one of 6 dietary treatments (control diet containing no supplemental minerals, 5 mg Mo/kg DM plus 0.2% S from MoS, 5 mg Cu/kg DM from $CuSO_4$, 5 mg Cu/kg DM from $CuSO_4$ plus 5 mg Mo and 0.2% S from MoS, 5 mg Cu/kg DM from CuLys, or 5 mg Cu/kg DM from CuLys plus 5 mg Mo and 0.2% S from MoS) to a basal diet containing 6.2 mg Cu/kg DM. Four milliliters of a solution containing 4 mg OVA in PBS and FIA was injected subcutaneously on d 7 and 77 of the study. Different animals per pen were injected on each day. Blood samples were taken prior to injection and 7, 14, and 21 d post injection. Serum samples were analyzed for antibody titers specific to OVA using an Elisa procedure described by Droke and Loerch (1989).

They reported that treatment had no effect on antibody titer production specific for OVA on either day.

In some of the above mentioned studies, Cu had no effect on antibody titer concentrations specific to PRBC and OVA. It is unknown why there was no effect. It may be because an animal may need to be deficient or have excess amounts of Cu before an effect is seen.

Cu deficiency and toxicity

Copper deficiency and toxicity can result because of many factors. The effects of these factors can depend on the species and breed of the animal. For example, sheep are more susceptible to toxicities than cattle and pigs because they cannot excrete Cu in bile as efficiently as the two other species (Dameron and Howe, 1998). Deficiencies are more widespread and occur naturally in grazing ruminants (Underwood, 1981; McDowell, 1992). This may be caused by poor uptake of Cu by plants (McDowell, 1992) that could be caused by a lack of Cu in the soil or other minerals that will bind Cu and prevent its absorption into the plants (Underwood, 1981).

Another factor that can cause deficiencies is the chemical form of Cu. Different forms of Cu are more available to the animal. Copper carbonate ($CuCO_3$) is more available than Cu sulfate ($CuSO_4$) and $CuSO_4$ is more available that Cu oxide (CuO) (Allen and Solomons, 1984). If CuO were the only Cu source available to swine, they may become Cu deficient because CuO is poorly available to swine (Cromwell et al., 1989).

The availability of theses sources to the animal may also be affected by interactions between Cu and other minerals or substances. Minerals can have

antagonistic effects with each other if they are not fed in proper ratios. Two antagonistic minerals to Cu are molybdenum (Mo) and sulfur (S). These minerals by themselves if fed in high amounts can cause a Cu deficiency, but high S fed along with high Mo can exaggerate the interaction between Mo and Cu (McDowell, 1992). Ruminants are more susceptible to this interaction than non-ruminants because of the microorganisms in the rumen (McDowell, 1992). When sulfate enters the rumen, the microorganisms convert the sulfate to sulfide (Dick et al., 1975; Suttle, 1975; Allen and Solomons, 1984; McDowell, 1992). The sulfide will bind to Mo and form a thiomolybdate (Allen and Gawthorne, 1987, McDowell, 1992; Baker and Ammerman, 1995), which then binds to Cu. Tetrathiomolybdates (MoS_4^{2-}) are excreted from the body because they cannot be absorbed, while mono-, di-, and trithiomolybdates bind to albumin and are absorbed into the blood. Thiomolybdates then become tightly bound in the plasma and plasma Cu is increased (Dick et al., 1975; McDowell, 1992; Baker and Ammerman, 1995). Copper deficiency results because the Cu within thiomolybdates is highly insoluble and non-utilizable (McDowell, 1992; Dameron and Howe, 1998) and cannot be used by body tissues (McDowell, 1992).

Cu deficiency can also result when other antagonistic minerals, such as Fe and Zn (Allen and Solomons, 1984), cadmium (Hill and Starcher, 1965), silver (Hill et al. 1964), and lead (Klauder and Petering, 1977), are high in the diet. Also, high ascorbic acid (Allen and Solomons, 1984), high fiber (Allen and Solomons, 1984; Dameron and Howe, 1998), and high protein (McCall and David, 1961; Reinhold et al., 1967) in the diet can cause Cu deficiency. If these minerals and compounds listed here and above are low in

the diet or Cu is high in the diet, Cu toxicity can result. Deficiencies and toxicities can produce many problems in the body

Deficiency Consequences: Since Cu is incorporated into many enzymes in the body that result in Cu being important in many physiological functions, problems can occur in the body when Cu is deficient. Anemia is one of these problems (Underwood, 1981; McDowell, 1992; Baker and Ammmerman, 1995). There is an impairment of Fe mobilization (Baker and Ammerman, 1995) because ceruloplasm production is decreased in the liver (Owen, 1965; Harris and Disilvestro, 1981). Plasma Cu levels can be as low as 0.1 to 0.2 mg/ml.

Bone disorders can result from Cu deficiency (Underwood, 1981; McDowell, 1992; Dameron and Howe, 1998). Decreased Cu has been shown to cause bone disorders in cattle (Mills et al, 1976) and rats (Alfaro and Heaton, 1973) as well as in other species, although the occurrence of bone disorders is low in cattle (Underwood, 1981). Collagen and lysyl oxidase (Cu dependent enzymes) are both important in providing strength and stability for bone (Committee on Animal Nutrition et al., 1980; Underwood, 1981). Bone disorders can occur because of a decrease in lysyl oxidase and collagen production (each contain Cu; Committee on Animal Nutrition et al., 1980; Underwood, 1981). During Cu deficiency, collagen fails to undergo cross-linking and maturation (Harris and Odell, 1974). Lysyl oxidase is important in the conversion of lysine to desmosine, which is important in forming this cross-linking (Committee on Animal Nutrition et al., 1980; Underwood, 1981). Without this crosslinking, the stability and strength of collagen is decreased which causes a decrease in bone strength (Committee of Animal Nutrition et

al., 1980). Lameness can result from this during Cu deficiency. Copper deficient cattle have been seen to have swollen fetlocks/pasterns (Becker et al., 1965; Underwood, 1981).

Cardiovascular disorders can occur during Cu deficiency. Copper is important for elastin synthesis (Underwood, 1981). Elastin allows the veins and arteries to expand and contract without tearing. Lysyl oxidase is also important in this process. Lysyl oxidase is involved in the conversion of lysine to desmosine, which is needed for elastin to undergo cross-linking (Committee on Animal Nutrition et al., 1980; Underwood, 1981). This allows arteries and veins to be elastic. Problems can occur if the arteries cannot expand and contract. Because of this reduced elasticity (Committee on Animal Nutrition et al, 1980), aortic ruptures have occurred during a severe Cu deficiency (Underwood, 1981; Dameron and Howe, 1998).

Falling disease is a result of Cu deficiency in cattle (Bennett and Hall, 1939; Underwood,1981; McDowell, 1992; Dameron and Howe, 1998) when cattle have plasma Cu concentrations as low as 0.01 to 0.02 mg/dl and liver Cu concentrations around 2 mg Cu/kg DM (Underwood, 1981). Falling disease occurs when cattle die suddenly caused by acute heart failure (McDowell, 1992). There are small lesions that form on the heart (Mills et al., 1976; Underwood, 1981; McDowell, 1992) that cause a slow but progressive degeneration of the myocardium with replacement fiber (Underwood, 1977). Cardiovascular lesions have also been seen in mice (Rowe et al., 1977), rats (Petering et al., 1986), rabbits (Hunt and Carlton, 1965), and pigs (Ganezer et al., 1976).

Copper deficiency has been shown to cause achromotrichia or discoloration of hair and wool (Underwood, 1981; Blakely and Hamilton, 1985; McDowell, 1992) because of a decrease in tyronsinase (McDowell, 1992). Tyrosinase is important in

converting tyrosine to melanin. Sheep are very sensitive to Cu deficiency, which results in bands of varying colors being produced easily in wool (Underwood, 1981). Underwood (1981) stated that in black wool black color is blocked after only two days of deficiency. Keritinization of hair and wool is also affected by Cu deficiency (Underwood, 1981; Blakely and Hamiliton, 1985; McDowell, 1992). Problems occur because Cu is important for the formation or for incorporation into disulfide groups that provide cross-linkages or bonding of keratin (McDowell, 1992). There is a decrease in growth and appearance of wool (Suttle and Angus, 1976). Tensil strength and elastin production are decreased because of a decrease in lysyl oxidase (Underwood, 1981). The result is a thin, harsh coat (Dameron and Howe, 1998). Wool becomes straight, steely, stringy, and has no crimp (Underwood, 1981).

Copper deficiency can cause swayback or neonatal ataxia in lambs (Bennett and Chapman, 1937; Committee on Animal Nutrition, 1980; Underwood, 1981; McDowell, 1992). Swayback causes spastic paralysis (Underwood, 1981; McDowell, 1992). This is an incoordination of the hind legs where the animal walks with a stiff gait (McDowell, 1992). They also exhibit an exaggerated swaying of the hind quarters (NRC, 1985). This central nervous system (CNS) disorder is believed to be linked to a maternal Cu deficiency during the development of CNS (Underwood, 1981; McDowell, 1992). Some lambs are paralyzed at birth and die immediately, while other lambs are born weak, are unable to nurse, and eventually die (McDowell, 1992). This disease is believed to be caused by incomplete myelin formation or degeneration of myelin sheath of nerve fibers (myelin aplasia; Underwood, 1981; McDowell, 1992). It is believed that not enough energy is being produced to make phospholipids because cytochrome C oxidase (a Cu

dependent enzyme important in energy production is deficient in the brain (Howell and Davison, 1959) and spinal cord (Barlow, 1963). Myelin aplasia will result from a lack of myelin (Mills and Williams, 1962) and also will result in degeneration of motor neurons by the brain and spinal cord (Underwood, 1981).

Copper deficiency has been shown to cause reproductive problems (Underwood, 1981; McDowell, 1992). Estrus can be delayed or depressed in animals (McDowell, 1992). Calving difficulties and retained placentas have been shown to occur during deficiency (McDowell, 1992). The fetus can also be born dead or calves can have congenital rickets because the mother was Cu deficient during the calf's development (McDowell, 1992).

The immune system has also been shown to be affected by Cu deficiency. There has been a decrease in immune function with T-cell, B-cell, neutrophils, and macrophage production decreased (McDowell, 1992). In mice, antibody producing cells have been shown to be decreased during deficiency (Prohaska et al., 1983). Sheep tend to have a decreased resistance to infection (Woolliams et al., 1986).

Toxicity Consequences: Toxicity occurs when tissue levels of Cu are elevated. Ruminants are most sensitive to toxicity (McDowell, 1992). In cattle, a concentration of 100 mg Cu/kg DM in the diet is the tolerance level with concentrations between 100 and 400 mg Cu/kg DM being toxic (McDowell, 1992). Sheep are more susceptible to Cu toxicity than cattle (Underwood, 1981). Toxicity levels in sheep are approximately 40 to 50 mg Cu/kg in the diet (NRC, 1985). In sheep, excretion of Cu in bile is minimal (Dameron and Howe, 1998). Dameron and Howe (1998) stated that this might be why sheep can only tolerate low levels of Cu in the diet and body before toxicity occurs.

Copper poisoning can result because other minerals (antagonists) that can bind Cu, such as Mo, are low (Dick and Bull, 1945). There are many symptoms of Cu toxicity. They can either be acute or chronic symptoms. Animals can suffer nausea and vomiting (Allen and Solomons, 1984; McDowell, 1992). McDowell (1992) states that other symptoms of Cu poisoning are increased salivation, abdominal pain, convulsions, and collapse. Anemia, muscular dystrophy, decreased growth, and impaired reproduction can result because of toxicity (NRC, 1980), as well as jaundice, hepatic necrosis, and intravascular hemolysis that can be fatal (Allen and Solomons, 1984). If liver Cu concentrations reach 1000 mg Cu/kg DM, Cu may be released into the blood and increase blood values 10 times that of normal values, which results in hemolytic crisis and ultimately death (Underwood, 1981).

Cu toxicity can be prevented. Proper ratios of minerals need to be maintained in the diet. For example, if Cu is increased significantly in the diet without increasing Zn, toxicity could result in the animal because there are fewer substances available to bind excess Cu. Zinc can be used to protect against Cu toxicity. Zinc decreases storage of Cu in the liver (Pope, 1971), but this is seen mostly in monogastrics (McDowell, 1992). Metallothionine, a zinc-containing molecule, can also help prevent Cu toxicity. Metallothionine has a higher affinity for Cu than for Zn. Copper can displace Zn on metallothionin and bind up to 12 Cu molecules (Dameron and Howe, 1998) decreasing the likelihood of toxicity.

Organic verses inorganic sources of copper

In ruminants, Cu bioavailability is low (Spears, 1996). Spears (1996) stated that providing a form of Cu that would avoid interactions and remain more stable in the

rumen and abomassum might be beneficial because the mineral will be unable to bind to antagonists or other molecules that may prevent Cu from being utilized by various systems in the body. Copper's affects on the body may be dependent on the source and level of Cu being used (Engle, 2001). There have been debates over whether organic or inorganic sources of Cu are more bioavailable to the animal. Spears (1996) stated that there is a theory that organic sources of minerals were more bioavailable than inorganic sources because the organic sources are more similar to biologically active forms of minerals in the body and in feed. Organic forms of minerals may prevent binding of antagonists or other molecules in the body because they are already bound to a molecule and have less or no binding sites available. Also, animals fed inorganic sources of minerals have to first convert these minerals to biologically active forms before the body can utilize them (Spears, 1996).

Traditionally, Cu has been supplemented in inorganic forms and are fed in the form of mineral salts. Some forms of inorganic sources of Cu are Cu sulfate ($CuSO_4$), Cu chloride ($CuCl_2$), cupric oxide (Cu_2O), Cu oxide (CuO), and Cu carbonate ($CuCO_3$). Copper sulphate is the most widely used inorganic form of Cu in diets of animals.

Spears (1996) reported that in recent years there has been increased interest in using organic forms of Cu because of improved growth, reproduction, and health seen in various studies. Organic forms of Cu are often bound to an amino acid or protein in various types of complexes, chelates, or proteinates (Spears, 1996). Complexes are formed when a ligand (amino acids, peptides, polypetides) binds to a metal ion. An example of a complex is Cu lysine (CuLys). Chelates are complexes between a ligand and a metal ion. Kratzer and Vohra (1996) stated that to be classified as a chelate the

ligand must contain a minimum of two functional groups (oxygen, nitrogen, amino, or hydroxyl) that can donate a pair of electrons to be able to bind to the metal ion. The ligand must also form a heterocyclic ring structure with the metal. Chelates are often composed of a variety of minerals rather than just one type of metal. Proteinates are metals that are chelated to amino acids and/or partially hydrolyzed proteins. An example of a proteinate is Cu proteinate (CuProt).

Results tend to vary among experiments in the availability of Cu in the organic verses the inorganic forms. In studies using rats, Kirchgessner and Grassmann (1970) fed the rats a low Cu diet for 3 to 4 weeks. The rats were then supplemented with 3.18 mg Cu/kg DM. Copper was supplemented as $CuSO_4$ (inorganic Cu source) or Cu amino acid, Cu peptide or Cu polypeptide complex (organic Cu sources) for 14 d. The results showed that absorption rate was greater in rats fed organic forms of Cu than in rats fed inorganic sources of Cu (Kirchgessner and Grassmann, 1973). Kincaid et al. (1986) conducted a study using 30 heifer and 15 bull holstein calves. The calves were fed a basal diet composed of barley and hay. The calves were supplemented with Cu proteinate (CuProt) or $CuSO_4$ in a pellet form or given no supplemental Cu. The pellet including either CuProt or $CuSO_4$ contained 19 mg Cu/kg DM. The unsupplemented concentrate mix contained 5 mg Cu/kg DM and the hay contained 1 mg Cu/kg DM. Liver and plasma Cu concentrations were measured. Kincaid et al. (1986) reported that the organic source of Cu (CuProt) was more available than the inorganic Cu source ($CuSO_4$).

Other studies have shown that organic sources of Cu were not more available than inorganic sources of Cu. Baker et al. (1991) fed 180 New Hampsire x Columbian

crossbred male chicks a basal diet composed of corn and soybeans that was fortified with 275 mg Cu/kg DM from $CuSO_4$ that contained a total of 290 mg Cu/kg DM. The chicks were supplemented with 75 or 150 mg Cu/kg DM from $CuSO_4$, Cu-lysine (CuLys), cupric oxide (Cu_2O), or Cu oxide (CuO) or received not supplemental Cu. They reported that availability of Cu from organic sources of Cu was not different than inorganic sources of Cu. Similar results were shown by Aoyogi and Baker (1993). Eighty one day old New Hampsire x Columbian crossbred male chicks were fed a Cu deficient casein-soy concentrate basal diet that contain 0.56 mg Cu/kg DM. The chicks were supplemented with 0, 0.5, and 1 mg Cu/kg DM from $CuSO_4$ or 0.5 and 1 mg Cu/kg DM from CuLys. At the end of the study, chicks were killed and bile was removed from the gall bladder and analyzed for Cu. Results showed that organic sources of Cu were no more available than inorganic sources of Cu.

Performance: Previous studies evaluating Cu supplementation to growing diets fed to ruminants have yielded inconsistent results whether there was a difference between organic or inorganic sources of Cu on performance. Ward et al. (1993), as mentioned earlier, supplemented 5 mg Mo/kg DM plus 0.2% S from MoS, 5 mg Cu/kg DM from $CuSO_4$, 5 mg Cu/kg DM from $CuSO_4$ plus 5 mg Mo and 0.2% S from MoS, 5 mg Cu/kg DM from CuLys, or 5 mg Cu/kg DM from CuLys plus 5 mg Mo and 0.2% S from MoS, or no supplement (the basal diet contained 6.2 mg Cu/kg DM) to 126 Angus and Angus x Hereford crossbred steers. They reported that steers supplemented with an inorganic Cu source ($CuSO_4$) had greater ($P < 0.01$) gains and tended to have greater ($P < 0.10$) feed efficiency (FE) than steers supplemented with an organic Cu source (CuLys; Ward et al., 1993). They also observed no treatment effects on ADG and ADFI (Ward et al., 1993).

In 2000, Engle and Spears conducted a study where they fed 36 Angus and 24 Hereford-Angus crossbred steers a corn silage based growing diet that contained 10.2 mg Cu/kg DM. They supplemented the steers 20 or 40 mg Cu/kg DM from $CuSO_4$, or 20 mg Cu/kg DM from Cu citrate, Cu Prot, or tribasic Cu chloride, or gave no supplemental Cu. They reported no significant differences in performance among steers supplemented with either organic or inorganic Cu sources. During the finishing phase of this same trial where a high concentrate diet containing 4.9 mg Cu/kg DM was fed, Engle and Spears (2000) supplemented the same steers with the same treatments. Performance in the finishing phase was also not affected by Cu source. Similar results were seen in a study by Rabiansky et al. (1999). They supplemented 40 Brahman x Hereford crossbred heifers with 8 or 16 mg Cu/kg DM from $CuSO_4$ or CuLys or no supplemental Cu to a basal diet containing 28 mg Cu/d. Copper source had no effect on performance.

Liver/Plasma Cu status: Organic and inorganic Cu source effects on liver and plasma Cu concentrations seem to vary. Kincaid et al. (1986) conducted a study using 30 heifer and 15 bull Holstein calves. The calves were fed a basal diet composed of barley and hay. The calves were supplemented with 19 mg Cu/kg DM from either Cu proteinate (CuProt) or $CuSO_4$ in a pellet form or given no supplemental Cu. The unsupplemented concentrate mix contained 5 mg Cu/kg DM and the hay contained 1 mg Cu/kg DM. The calves that were supplemented with the organic Cu source (CuProt) had significantly greater liver Cu concentrations than calves supplemented with the inorganic Cu source ($CuSO_4$). Plasma Cu concentrations were also greater ($P < 0.05$) in calves supplemented with the organic Cu source than the inorganic Cu source (Kincaid et al., 1986). Engle and Spears (2000) reported that steers supplemented with the organic Cu source (Cu

proteinate) tended to have greater liver Cu concentrations than steers supplemented with the inorganic Cu source ($CuSO_4$). Plasma Cu concentrations were unaffected by Cu source (Engle and Spears, 2000). In contrast, Stanton et al. (1998) reported higher ($P < 0.05$) liver Cu concentrations in steers that were supplemented with $CuSO_4$ relative to steers supplemented with iso-amounts of the organic Cu (Cu amino acid complex) sources.

Availability studies comparing organic Cu sources to inorganic Cu sources have been studied more extensively in non-ruminant species, such as rats and chicks. Du et al. (1996) conducted a study using 60 male Sprague-Dawley rats. The rats were supplemented with 0 or 1000 mg Zn/kg DM or 5 or 15 mg Cu/kg DM from $CuSO_4$, CuProt, or CuLys to a basal diet containing 0.81 mg Cu/kg DM. Rats supplemented with an organic Cu source (CuProt) had greater ($P < 0.05$) liver Cu concentrations than rats supplemented with and inorganic Cu ($CuSO_4$) source (Du et al., 1996). In a second experiment, Du et al. (1996) used the same number and type of rats, the same diet and treatments, except Zn was replaced with Fe. They reported that overall liver Cu concentrations were again greater ($P< 0.05$) when rats were supplemented with an organic Cu source (CuLys) than when supplemented with an inorganic Cu source ($CuSO_4$).

Immune function: Hutcheson (1989) and Spears (2001) indicated that organic Cu sources might be more available when an animal is stressed (for example by transport, illness, and other factors). Illness can put tremendous amount of stress on the body and cause the body to try to rid itself of the illness. Increased excretion of minerals in urine during stress has been shown (Orr et al., 1990) Nockels et al. (1993) reported that when

supplementing CuLys or $CuSO_4$ to eight Charolais crossbred steer calves, upon feed and water removal (stressor), CuLys was retained more in the body than $CuSO_4$. This could explain why organic sources of Cu are believed to be more available than inorganic sources of Cu during a stressed period.

Results from studies have varied. Nockels (1991) supplemented Cu Prot and $CuSO_4$ to steers found that antibody titers to IBR were higher in cattle that were fed the organic Cu source than the cattle fed the inorganic Cu source. In contrast, Ward et al. (1993) reported no treatment effect on antibody titer concentration to OVA and cell mediated immune response to PHA when supplementing $CuSO_4$ or CuLys to steers. The differences in results between studies is unknown but could be caused by breed differences, environmental differences, source differences, or even the branch of the immune system being studied.

Summary

Because of its incorporation into many enzymes, Cu is a part of many physiological functions in the body. Some of these physiological functions are Fe metabolism, elastin and collagen formation and immune function. If Cu were not present in the diet, Cu deficiency would occur. Cu deficiency can cause depigmentation of hair and wool, bone problems, cardiac lesions, and even death. High amounts of Cu in the diet can cause Cu toxicity that can lead to death. Many studies have been conducted testing the effects of Cu on the body under many different scenerios. Recently, research has begun to focus on whether inorganic or organic sources of Cu are more available to animals. Because results have been inconsistent, more research is needed in order to determine the effects of Cu on the body.

Chapter 3

MATERIALS AND METHODS

Prior to the initiation of this experiment, care, handling, and sampling of animals herein were approved by the Colorado State University Animal Care and Use Committee.

Forty-eight purebred Angus steers (approximately 7 months of age, 220 + 9.1 kg) from the Colorado State University Beef Improvement Center in Saratoga, WY, were transported approximately 230 km to the Colorado State University feedlot facilities outside of Fort Collins, CO. Previously, all calves grazed the same pastures and were under the same management.

Growing phase

Upon arrival, steers were weighed on two consecutive days, implanted with Ralgro® (Intervet, Millsboro, DE), vaccinated with Cattle Master® 4 and Bovishield® 4+/L5 (Pfizer Animal Health, Exton, PA), and dewormed with Dectomax® (Pfizer Animal Health, Exton, PA). A liver biopsy was also taken and analyzed for Cu. After a two-week adjustment period, steers were stratified by body weight and liver Cu concentrations and randomly assigned to groups. Groups were then randomly assigned to treatments. Treatments consisted of: 1) control (no supplemental Cu), 2) 10 mg Cu/kg DM from Cu sulfate ($CuSO_4$), 3) 10 mg Cu/kg DM from a Cu amino acid complex (Availa Cu; Zinpro Corporation, Eden Prairie, MN), 4) 20 mg Cu/kg DM from $CuSO_4$, and 5) 20 mg Cu/kg DM from Availa Cu. Steers were housed in individual pens (2 m x

12.5 m) equipped with automatic waterers and fed a corn-alfalfa based growing diet for 56 d (Table 1). The basal diet was formulated to meet or exceed all nutrient requirements for growing steers with the exception for Cu (NRC, 1996). The basal diet contained 7.1 mg Cu/kg DM, 32.8 mg zinc (Zn)/kg DM, 68.1 mg iron (Fe)/kg DM, 0.135% sulfur (S), and 0.59 mg molybdenum (Mo)/kg DM. Diets were fed at 0700 h in amounts that would allow ad-libitum access to feed throughout the day. Over the 56 d growing phase, daily feed offerings were recorded. Feed refusals and body weights were measured every 28 d in order to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (FE).

Blood samples were taken via jugular venapuncture in trace mineral free heparinized vacutainer tubes every 28 d (Becton Dickenson Co., Franklin Lakes, NJ). On d 0 and 56, a blood sample was taken via jugular venapuncture in trace mineral free unheparinized vacutainer tubes and a second blood sample was taken in a heparinized vacutainer tube to be stored as whole blood. Also on d 0 and 56, red blood cell (RBC) superoxide dismutase (SOD) enzyme activity was determined. Samples were stored on ice and immediately transported to the laboratory to be processed (described below).

On d 56, liver biopsies were obtained from each steer. A small area on the right side of the steer around the 11^{th} and 12^{th} rib was clipped of hair, scrubbed three times with iodine, and then with 70 % alcohol. Three milliliters of a two percent lidocaine solution (Abbott Laboratories, Chicago, IL) was injected between the 11^{th} and 12^{th} rib on a line made from the tubercoxae to the tip of the shoulder and an incision was made with a #11 scalpel blade. A core sample of liver was taken using the "Tru-Cut" technique (Pearson and Craig, 1980) as modified by Engle and Spears (2000b). Briefly, a modified

Jam Shide bone marrow punch (0.5 cm in diameter and 14 cm in length; Sherwood Medical, St. Louis, MO) was used. Samples were then immediately rinsed with 0.01 M phosphate buffered saline (PBS) solution and placed into an acid washed polyethylene tube, capped, and stored on ice until it was brought back to the laboratory. Samples were then stored at –20°C until analyzed for Cu.

Humoral immune response was measured on d 28 by injecting 5 milliliters of a 25% pig red blood cell (PBRC) solution into the neck muscle of each steer (Engle et al., 1999). Blood samples were taken via jugular venapuncture in unhepranized vacutainer tubes prior to injection and on d 7, 14 and 28-post injection. Samples were stored on ice and transported to the laboratory to be processed.

On d 56, an in-vivo assessment of cell-mediated immune response was conducted (Fritz et al., 1990). Briefly, a small square on the left side of the steer, immediately posterior to the scapula, was clipped of hair. One hundred and fifty micrograms of phytohemagglutinin (PHA; Cat. No. L8754, Sigma Chemical, St. Louis, MO) in 0.1 ml of 0.01 M PBS was injected intradermally into two sites approximately one inch apart. The injected sites were measured in millimeters using skin fold calipers (Vernier Type 6914; Scienceware, Bel Art Products, Pequannock, NJ) prior to injection and at 8, 12, 24, and 48 h post injection. Inflammatory response was measured as a change in skin thickness. The average of the two sites on each steer were used for statistical analysis.

Finishing Phase

Steers were fed the same dietary treatments that they received in the growing phase, however, the basal diets where replaced gradually (over a 10 d period) with a high concentrate finishing diet (Table 1). Diets were formulated to meet or exceed all nutrient

requirements for finishing steers with the exception for Cu (NRC, 1996). The basal diet contained 6.1 mg Cu/kg DM, 42.3 mg Zn/kg DM, 49.7 mg Fe/kg DM, 0.19% S, and 0.42 mg Mo/kg DM. Feed refusals were measured as mentioned in the growing phase. Steers were reimplanted with Revalor® IS (Intervet, Millsboro, DE) on d 28. Steers were weighed and blood samples were collected every 28 d for plasma Cu analysis and on d 84 for serum IgG and IgM. Superoxide dismutase activity and total hemoglobin was measured on d 84 and liver biopsies were obtained on d 112. All samples were collected, stored, and analyzed as mentioned before.

Using 4 steers/treatment, secondary immune response was measured on d 84. Five milliliters of 25% PBRC were again injected into the neck muscle. Blood samples were collected prior to injection and 7, 14, and 21 d-post injection and processed and stored as mentioned previously. Using the remaining steers (5 steers/treatment), a primary humoral immune response to a second antigen was measured according to a procedure described by Ward et al. (1993) with a few modifications. Two milliliters of a solution containing 106 mg of ovalbumin (OVA; Sigma A5503), 60 mL Freund's Incomplete Adjuvant (FIA; Sigma F-5506), and 60 mL of phosphate buffered saline (PBS) were injected subcutaneously and one milliliter was injected intradermally to give a total injection of 4000 ug of OVA/animal. Blood samples were collected via jugular venapuncture in unheparinized vacutainer tubes (Becton Dickenson Co., Franklin Lakes, NJ) prior to injection and 7, 14, and 21 d post-injection. Samples were stored on ice and transported to the laboratory to be processed.

Analytical Procedures

Blood preparation. All blood samples were centrifuged at 1200 x *g* for 25 min. Plasma and serum from blood samples taken in trace mineral free vacutainer tubes were harvested and stored in an acid washed polyethylene tube until analyzed for plasma Cu concentrations and serum immunoglobulin G (IgG) and immunoglobulin M (IgM) concentrations. One milliliter of the RBC layer from the heparinized vacutainer tubes was lysed in 4 mL of cold dH_2O in an acid washed polyethylene tube and was analyzed for SOD activity. Serum samples for PRBC antibody titer analysis were stored in polystyrene tubes. Serum samples for OVA antibody titer analysis were divided equally into three tubes and were heated for 30 min at 56°C. All samples were stored at -20°C.

Plasma and Liver Cu. One mL of 10% trichloroacetic acid (TCA) was added to one mL of plasma or standard and then vortexed vigorously. The mixture was placed in a –20°C freezer for 30 minutes to aid in precipitation and then centrifuged at 1200 x *g* for 10 minutes. The supernatant was removed to a clean tube and was diluted in dH_2O to fit within a linear range of a standard curve generated by linear regression of known Cu concentrations. Liver tissue samples were dried at 80°C for 24 h. Samples were weighed, placed into crucibles, and then dry ashed at 600°C overnight. Two milliliters of 3.6N nitric acid were added to the ashed samples. The crucibles were placed into an Ultrasonic Cleaner (Cole Palmer Model 8845-4) to aid in sample digestion. Samples were then diluted in dH_2O to fit within a linear range of a standard curve generated by linear regression of known Cu concentrations. Liver and plasma samples were read at 324.7 nm using a flame atomic absorption spectrophotometer (Varian Model 1275;

Walnut creek, CA, 94598). Concentrations of the samples were then determined by a standard curve generated by linear regression using standards of known concentrations.

Superoxide dismutase enzyme activity. Lysed RBC were analyzed for SOD activity using SOD 525™ Assay Kit (Biotech® 21010; Oxis Health Products, Inc., Portland, OR). The SOD activity was expressed as SOD activity per milligram hemoglobin (Sigma 525-A).

Serum IgG and IgM. Total IgG and IgM concentrations were determined using single radioimmunodiffusion assay kits (Stable et al. 1993; VMRD 240-30 and 246-30; Pullman, WA). Briefly, three microliters of serum were added to a well on a single radial immunodiffusion (SRID) plate that contained a monospecific antisera specific for either IgG or IgM. The plates were covered and were left undisturbed for 18 to 24 hours at room temperature. When the serum diffused into the gel that contained the antisera, a ring of precipitation formed that was proportional to the concentration of IgG or IgM in the serum. The diameter of the rings that formed was measured with a ruler in millimeters. These measurements were then compared to standards of known concentrations.

Pig red blood cell antibody titers. Samples were centrifuged at 1200 x *g* for 25 minutes, serum harvested and transferred to polystyrene tubes and were stored at –20°C until analyzed for PBRC antibody titers. PBRC antibody titers were measured using a microtiter hemagglutination assay (Ferket and Qureshi, 1992) to determine total immunoglobulin (Ig), immunoglobulin G (IgG), and immunoglobulin M (IgM) concentrations specific for PRBC. Total Ig is IgG concentrations and IgM concentrations combined.

Ovalbumin antibody titers. Samples were analyzed for antibody titers specific to ovalbumin using an Elisa procedure described by Engvall and Perlmann (1972).

Statistical analysis

Statistical analysis of data was performed using GLM procedure of SAS (2001). The model included treatment, time, and treatment x time. When treatment tended to be significant ($P < 0.20$), differences among means were determined using preplanned single degree of freedom contrasts. The contrast statements were as follows: 1) control vs Cu treatments, 2) 10 mg Cu/kg DM vs 20 mg Cu/kg DM, 3) 10 mg Cu/kg DM from $CuSO_4$ vs 10 mg Cu/kg DM from Availa Cu, and 4) 20 mg Cu/kg DM from $CuSO_4$ vs 20 mg Cu/kg DM from Availa Cu.

Chapter 4

RESULTS AND DISCUSSION

One steer from the 10 mg Cu/kg DM from $CuSO_4$ treatment died early in the study from bloat. All data collected from this steer were removed from statistical analysis.

Performance. Steer performance is reported elsewhere (Lee et al., 2000). Briefly, during the 56 d growing phase, steers supplemented with 20 mg Cu/kg DM had heavier ($P < 0.02$) body weights than steers supplemented with 10 mg Cu/kg DM. Steers supplemented with 20 mg Cu/kg DM from $CuSO_4$ had greater ($P < 0.04$) ADG and feed efficiency than steers supplemented with 20 mg Cu/kg DM from Availa Cu. During the finishing phase, steers supplemented with 10 mg Cu/kg DM from Availa Cu had heavier ($P < 0.01$) body weights than steers supplemented with 10 mg Cu/kg DM from $CuSO_4$. The results suggest that Cu source and concentration may have had an effect on performance. Previous studies evaluating Cu supplementation to growing and finishing diets have yielded inconsistent results (Ward et al., 1993; Ward and Spears, 1997; Engle and Spears, 2000; Engle and Spears, 2001). The reason for these conflicting performance results in cattle consuming growing and finishing diets is unclear. There are many factors that could potentially have an affect on the animals response to Cu supplementation such as the Cu concentration of the basal diet, the duration of Cu

supplementation, the absence or presence of dietary Cu antagonists (S, Mo, and Fe), environmental and health factors, and breed differences in Cu metabolism.

Copper Status. There was a treatment x time interaction for liver Cu concentrations. Therefore data was analyzed as a function of time (Table 2). Initial liver Cu concentrations were similar ($P > 0.15$) across treatments due to experimental design. On d 56 of the growing phase and d 112 of the finishing phase, control steers had lower ($P < 0.01$) liver Cu concentrations than steers supplemented with Cu, and steers supplemented with 20 mg Cu/kg DM had higher ($P < 0.01$) liver Cu concentrations than steers supplemented with 10 mg Cu/kg DM. On d 112, steers supplemented with 20 mg Cu/kg DM from Availa Cu had higher ($P < 0.01$) liver Cu concentrations than steers supplemented with 20 mg Cu/kg DM from $CuSO_4$ indicating a difference in availability of the different Cu sources. Similar to the present study, calves supplemented with an organic Cu source (Cu proteinate (CuProt)) had significantly greater liver Cu concentrations than calves supplemented with an inorganic Cu ($CuSO_4$) source (Kincaid et al., 1986). Engle and Spears (2000) reported that steers supplemented with the organic Cu source (Cu proteinate) tended to have greater liver Cu concentrations than steers supplemented with the inorganic Cu source ($CuSO_4$). This may suggest that the organic Cu source may be more bioavailable than the inorganic Cu source at certain concentrations. In contrast to the present study, Stanton et al. (1998) reported higher ($P < 0.05$) liver Cu concentrations in steers that were supplemented with $CuSO_4$ relative to steers supplemented with iso-amounts of the organic Cu (Cu amino acid complex) sources.

Availability studies comparing organic Cu sources to inorganic Cu sources have been studied more extensively in non-ruminant species, such as rats and chicks. In two studies using male chicks, Guo et al. (2001) found that organic Cu sources (CuLys, Cu amino acid chelate, and CuProt A, B, and C) had similar effects on liver Cu concentrations as inorganic Cu source ($CuSO_4$). Rats supplemented with an organic Cu source (CuProt) had greater ($P < 0.05$) liver Cu concentrations than rats supplemented with an inorganic Cu source ($CuSO_4$; Du et al., 1996). In a second experiment, Du et al. (1996) reported that overall liver Cu concentrations were again greater ($P< 0.05$) when rats were supplemented with an organic Cu source (CuLys) than when supplemented with an inorganic Cu source ($CuSO_4$). The reason for the discrepancies between these trials is unknown. Environment, antagonistic effects from other minerals, breed and species of animal, and source and concentration of Cu could play a role in these variations.

In the present study, liver Cu concentrations of less than 20 mg of Cu/kg DM (Mills, 1987) were considered deficient. Control steers in this study decreased slightly below 20 mg of Cu/kg DM by d 56 of the growing phase but increased above the deficiency level by d 112.

There was no time or treatment x time interaction for plasma Cu. Therefore, overall plasma Cu concentration means are shown in Table 3. Initial plasma Cu concentrations were similar ($P > 0.15$) across treatments. On d 56 of the growing phase, control steers tended to have lower ($P < 0.06$) plasma Cu concentrations than steers supplemented with Cu. Steers supplemented with 20 mg Cu/kg DM had greater ($P < 0.04$) plasma Cu concentrations than steers supplemented with 10 mg Cu/kg DM. On both d 56 of the growing phase and overall in the finishing phase, steers supplemented

with 20 mg Cu/kg DM from Availa Cu had greater ($P < 0.03$) plasma Cu concentrations than steers supplemented with 20 mg Cu/kg DM from $CuSO_4$. Plasma Cu concentrations of control steers were above concentrations considered to be deficient (less than 0.6 mg of Cu/L) throughout the entire trial (Mills, 1987).

During the finishing phase, steers supplemented with 20 mg Cu/kg DM tended ($P < 0.11$) to have greater plasma Cu concentrations than steers supplemented with 10 mg Cu/kg DM. The reason plasma Cu concentrations did not decrease in the control steers over time may be due to the liver's ability to release Cu into the blood in order to maintain blood Cu concentrations (Wiske et al., 1992). Plasma Cu concentrations tend to remain relatively stable until liver Cu concentrations are severely deficient (< 40 mg/kg Cu in liver) (Wiske et al., 1992). In trials supplementing 2 year old beef heifers ($CuSO_4$) (Gengelbach et al., 1994), Simmental, Charolais, and Angus calves ($CuSO_4$ and CuLys) (Kegley and Spears, 1994), Holstein bull calves ($CuSO_4$) (Ward et al., 1997), Holstein heifers ($CuSO_4$) (Torre et al., 1995), and gestating beef cattle ($CuSO_4$ and $CuCO_3$) (Niederman et al., 1994), steers supplemented with Cu had greater ($P < 0.05$) plasma Cu concentrations overall than control steers, which is similar to the present study. Engle and Spears (2001) reported similar results in a study supplementing $CuSO_4$ to Simmental steers. In contrast to the present study, Ward et al. (1993) when supplementing $CuSO_4$ and CuLys found no differences in plasma Cu concentrations among supplemented and unsupplemented steers. Furthermore, Kegley and Spears (1994) reported no differences in plasma Cu concentrations in steers supplemented with Cu oxide (CuO) and $CuSO_4$.

SOD enzyme activity. There were no treatment effects or treatment by time effects on SOD enzyme activity in both the growing and finishing phase (data not

shown). Similar to the present trial, Ward et al. (1993) found no difference among treatments in SOD activity when supplementing $CuSO_4$ or $CuCO_3$ verses no supplementation to steers. No difference in SOD activity was also observed when supplementing Holstein heifers with $CuSO_4$ (Torre et al., 1995) and when supplementing $CuSO_4$ or $CuCO_3$ to gestating beef cattle (Niederman et al., 1994) relative to unsupplemented cattle. The lack of a decrease in SOD activity in control steers most likely indicates that the basal diet Cu concentration was not low enough in Cu to produce a Cu deficiency. Paynter (1987) stated that SOD in RBC is one of the last enzymes that is affected by Cu deficiency. In the present study, steers were not depleted of Cu by dietary means. In studies where animals are fed semi-purified diets deficient in Cu, SOD activity is typically decreased relative to supplemented animals. Lambs that were depleted of Cu by adding molybdenum and sulfur to the diet had a 40% reduction in RBC SOD activity (Andrewartha and Caple, 1980). In a separate study using deficient lambs that grazed a pasture known to be deficient in Cu, SOD activity increased when lambs were fed a diet adequate in Cu (Andrewartha and Caple, 1980). Sheep (Jones and Suttle, 1981) and steers (Xin et al., 1991) fed severely Cu deficient diets (sheep plasma Cu concentrations < 0.5 mg/L; and steers plasma Cu concentrations < 0.8 mg/ml and liver Cu concentrations < 19 mg/kg DM) had lower SOD activity ($P < 0.01$) than Cu supplemented sheep and cattle.

Based on liver Cu concentrations on d 0, 56, and 112, only d 56 liver Cu values fell below those considered adequate (> 20 mg Cu/kg DM) (Mills, 1987). However, plasma Cu concentrations were above concentrations considered deficient (< 0.6 mg

Cu/L) (Mills, 1987) on all sampling dates. This is most likely why SOD activity were similar across treatments.

Immune function. There was neither a treatment nor a treatment by time effect on total immunoglobulin G (IgG) and total immunoglobulin M (IgM) concentrations in serum in the growing and finishing phase (data not shown). Stabel et al. (1993) found total IgM and IgG were not affected by Cu status in Cu-adequate and Cu-deficient Holstein calves. Ward et al. (1997) also found that supplementing Cu ($CuSO_4$) to a diet deficient in Cu to heifers had no effect on total IgG concentration in the serum of their calves.

Cell mediated immune response to phytohemagglutinin (PHA) is shown in Table 4. Kuby (1994) stated that the cell-mediated branch of the immune system (cytotoxic T lymphocytes, natural killer cells, and macrophages) is responsible for ridding the body of intracellular pathogens, virus-infected cells, and tumor cells. When injecting PHA or any other antigen into the body, the cell-mediated branch of the immune system responds to destroy this foreign substance. In the present experiment after injecting PHA, steers supplemented with 20 mg Cu/kg DM had a greater ($P < 0.01$) overall skin swelling response to PHA than steers supplemented with 10 mg Cu/kg DM. In previous research, Holstein bull calves supplemented with 10 mg Cu/kg from $CuSO_4$ had a greater ($P < 0.06$) immune response to PHA than did control steers at 12 h and 24 h post injection (Ward et al., 1997). However, in another study, Ward et al. (1997) reported that, when supplementing 10 mg Cu/kg DM from $CuSO_4$ to a diet containing 4.5 mg Cu/kg DM to 2-year-old pregnant heifers, Cu supplementation produced a lower skin swelling response to PHA in the calves of the heifers. It is unknown why there are differences in results

between studies. The breed of cattle, stage of production, or even gender of the animal may play a role in these variations.

The humoral branch of the immune system involves the interaction of B-cells with antigen (Kuby, 1994). It involves the differentiation and proliferation of these B-cells into antibody secreting plasma cells and memory cells (Kuby, 1994). Humoral immune response specific to pig red blood cells (PRBC) is shown in Table 5 (total immunoglobulin (Ig), IgG, and IgM antibody titers to PRBC). In the present study during the growing phase, total Ig and IgG titers to PRBC were greater ($P < 0.01$) in steers supplemented with 10 mg Cu/kg DM from $CuSO_4$ than steers supplemented with 10 mg Cu/kg DM from Availa Cu. During the finishing phase, there was a treatment by time effect for IgG antibody titers to PRBC. On d 14 post injection of PBRC, steers supplemented with 20 mg Cu/kg DM had greater ($P < 0.01$) IgG antibody titers to PRBC than steers supplemented with 10 mg Cu/kg DM. On d 21 post injection of PRBC, control steers had greater ($P < 0.01$) IgG antibody titers to PRBC than Cu supplemented steers. Overall in the finishing phase, IgG antibody titers to PRBC were greater ($P < 0.03$) in control steers than Cu supplemented steers. Immunoglobulin M antibody titers specific for PRBC tended to be greater ($P < 0.09$) in steers supplemented with Cu than controls. Also, steers supplemented with 20 mg Cu/kg from Availa Cu tended to have greater ($P < 0.09$) IgM antibody titers specific for PRBC than steers supplemented with 20 mg Cu/kg DM from $CuSO_4$. In a previous study, contrasting results were found when 70 d old calves were supplemented with 10 mg Cu/kg DM from $CuSO_4$ to a diet containing 4.5 mg Cu/kg DM. There was not a treatment effect after primary injection, however, calves supplemented with Cu had less antibody titers than control steers 14 d (P

< 0.07) and 21 d ($P < 0.09$) post-secondary injection (Ward et al., 1997). Also, Angus bull calves supplemented with 5 mg Cu/kg DM from $CuSO_4$ to a diet containing 5.2 mg Cu/kg DM had less ($P < 0.07$) antibody titers to PBRC than control steers (Ward and Spears, 1999). The reasons for the discrepancies between experiments are unclear.

There was a treatment by time effect ($P < 0.04$) for antibody titer response to ovalbumin (OVA) antigen (Table 6). Steers supplemented with 20 mg Cu/kg DM had more ($P < 0.04$) antibody titers to OVA than steers supplemented with 10 mg Cu/kg DM on d 21. On both d 14 and d 21 post injection, steers supplemented 20 mg Cu/kg DM from Availa Cu had greater ($P < 0.03$) antibody titers to OVA than steers supplemented with 20 mg Cu/kg DM from $CuSO_4$. Similar results have been reported by Ward and Spears (1999) when supplementing 5 mg Cu/kg DM from $CuSO_4$ to a diet containing 5.2 mg Cu/kg DM. Immune response to OVA was greater in Angus bull calves supplemented with Cu than in control steers 7, 14, 21, and 28 d post injection ($P < 0.06$). In contrast, Ward et al. (1993) reported that treatment had no effect on antibody titer production specific for OVA when supplementing 5 mg/kg Cu from either $CuSO_4$ or CuLys to a diet containing 6.2 mg Cu/kg DM.

In the present study, antibody titer concentrations to PRBC seemed to be more affected by the inorganic Cu source, whereas the antibody titer concentration to OVA seemed to be affected more by the organic Cu source. The reasons for this difference are unknown. One reason may be the amount of "stress" that these antigens put on the body. Hutcheson (1989) stated that it is believed that when the body is faced with a "stressor", such as nutrition or environmental stressors, than organic Cu sources are most effective. The OVA solution that was injected into the steers contained Freund's incomplete

adjuvant (FIA). Freund's incomplete adjuvant is often used to take a less antigenic antigen (such as OVA) and make it more antigenic (Kuby, 1994). Freund's incomplete adjuvant also prolongs the antigenic persistence from days to weeks and induces granuloma formation that may be responsible for facilitating antigen processing and presentation and T-cell activation (Kuby, 1994).

Implications:

In the current trial, there was no treatment by time interactions on performance, but overall performance tended to be affected by Cu concentration and source. Liver Cu concentrations were increased by Cu concentration as well as source. The effects of Cu supplementation on the animal's ability to respond immunologically were variable and depend on the class of immune cell being studied. B-cell stimulated primary and secondary immune responses tended to be enhanced by Cu supplementation. When a more antigenic antigen was administered, steers supplemented with 20 mg Cu/kg DM had higher antibody titers specific to OVA than steers supplemented with 10 mg Cu/kg DM, and steers supplemented with 10 mg Cu/kg DM from Availa Cu had higher antibody titers to OVA than steers supplemented with 10 mg Cu/kg DM from $CuSO_4$. Further research is needed to determine the effects of Cu on growth and immunity of beef cattle.

Table 1. Ingredient composition of basal diet

Ingredient	Growing	Finishing
	----------%[a]----------	
Whole Corn	47	86
Alfalfa Hay	45	6
Protein Supplement	7	6.7
Vitamin Premix[b]	(0.02)	(0.03)
Mineral Premix[c]	+	+
Monensin[d]	-----	+
Protein Supplement Composition		
Sunflower Meal 32%	87.30	------
Ground Corn	-----	53.40
Limestone	7.62	13.34
Urea	6.54	17.25
Feather Meal	2.88	-----
Salt	2.27	2.29
Biofos	1.15	8.55
Niacin 98%	0.29	0.27
Tylan 40	0.25	0.18
Rumensin 80	-----	0.26
Dyna-K	0.16	7.08
Wheat Midd	-----	5.64
Sulfur Flo	-----	0.47

[a]Dry matter basis
[b]Contained per kilogram of diet: 6,000,000 IU of vitamin A, 1,520,000 IU of vitamin D, and 6, 600 IU of vitamin E.
[c]Provided per kilogram of diet: 30 mg of Zn as $ZnSO_4$, 20 mg of Mn as $MnSO_4$, 0.5 mg of I as EDDI, 0.1 of Co as $CoCO_3$, and 0.1 mg of Se as Na_2SeO_3 (was adjusted depending on dietary treatment)
[d] Provided 22 mg of monensin/kg of DM

Table 2. Influence of copper concentration and source on liver copper concentrations in growing and finishing steers, mg/kg DM

	Dietary Treatment					
		$CuSO_4$		Availa Cu		
Item	Control	10 mg Cu/kg DM	20 mg Cu/kg DM	10 mg Cu/kg DM	20 mg Cu/kg DM	SE
Growing Phase						
Day 0	30.9	31.8	26.2	32.4	27.1	13.3
Day 56[a, b]	17.8	104.1	183.4	122.3	210.3	17.9
Finishing Phase						
Day 112[a, b, c]	37.4	113.9	116.9	94.8	194.6	14.7

[a]Control vs Cu *(P < .01)*
[b]10 vs 20 *(P < .01)*
[c]20 $CuSO_4$ vs 20 Availa Cu *(P < .01)*

Table 4. Influence of copper concentration and source on immune response to phytohemagglutinin in growing and finishing steers, mm

	Dietary Treatment					
		$CuSO_4$		Availa Cu		
	Control	10 mg Cu/kg DM	20 mg Cu/kg DM	10 mg Cu/kg DM	20 mg Cu/kg DM	SE
Time, hrs						
0	0	0	0	0	0	0.36
8	4.6	4.0	4.7	4.2	4.9	0.36
12	3.5	3.5	4.4	3.8	4.4	0.36
24	3.0	2.4	2.9	2.5	2.9	0.36
48	1.5	0.8	1.5	1.3	1.7	0.36
Total[a]	2.5	2.1	2.7	2.4	2.8	0.17

[a]10 vs 20 *($P < 0.01$)*

Table 5: Influence of Cu concentration and source on immune response to pig red blood cell antigen in growing and finishing steers, log_2

	Dietary Treatment					
		$CuSO_4$		Availa Cu		
	Control	10 mg Cu/kg DM	20 mg Cu/kg DM	10 mg Cu/kg DM	20 mg Cu/kg DM	SE
TOTAL Ig						
Growing						
0	0	0	0	0	0	0.30
Overall[a]	2.8	3.0	2.9	2.5	2.8	0.15
Finishing						
0	1.0	1.0	1.0	1.4	1.0	0.44
Overall	3.0	3.1	2.8	2.9	3.3	0.22
IgG						
Growing						
0	0	0	0	0	0	0.23
Overall[a]	1.8	2.1	1.8	1.7	1.9	0.11
Finishing						
0	0	0	0	0	0	0.35
7	2.6	2.0	2.5	2.4	1.5	0.44
14[b, c]	2.6	2.0	3.0	2.4	3.8	0.35
21[d]	2.0	0.8	0.8	0.4	0.5	0.43
Overall[e, f]	1.8	1.2	1.6	1.3	1.4	0.18
IgM						
Growing						
0	0	0	0	0	0	0.26
Overall	1.0	0.9	1.1	0.8	0.9	0.13
Finishing						
0	1.0	1.0	1.0	1.4	1.0	0.49
Overall[g, h]	1.2	1.9	1.3	1.6	1.9	0.25

[a]10 $CuSO_4$ vs 10 Availa Cu ($P < 0.01$)
[b]10 vs 20 ($P < 0.01$)
[c]20 $CuSO_4$ vs 20 Availa Cu ($P < 0.17$)
[d]Control vs Cu ($P < 0.01$)
[e]Control vs Cu ($P < 0.03$)
[f]10 vs 20 ($P < 0.16$)
[g]Control vs Cu ($P < 0.09$)
[h]20 $CuSO_4$ vs 20 Availa Cu ($P < 0.09$)

Table 6. Influence of copper concentration and source on immune response to ovalbumin antigen in growing and finishing steers, log_{10}

	Dietary Treatment					
		$CuSO_4$		Availa Cu		
	Control	10 mg Cu/kg DM	20 mg Cu/kg DM	10 mg Cu/kg DM	20 mg Cu/kg DM	SE
Time, Day						
0	0	0	0	0	0	0.003
7[a, b]	0	0.002	0	0	0	0.011
14[c, d]	0.047	0.054	0.054	0.054	0.102	0.014
21[e, f, g]	0.054	0.067	0.072	0.064	0.132	0.016

[a]Control vs Cu ($P < 0.05$)
[b]10 vs 20 ($P < 0.02$)
[c]10 vs 20 ($P < 0.12$)
[d]20 $CuSO_4$ vs 20 Availa Cu ($P < 0.03$)
[e]Control vs Cu ($P < 0.11$)
[f]10 vs 20 ($P < 0.04$)
[g]20 $CuSO_4$ vs 20 Availa Cu ($P < 0.02$)

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