THESIS

INTERACTION OF COPPER SOURCE, BILE COMPOSITION ON MICROBIAL GROWTH, AND CU PROTEIN HOMEOSTASIS

Submitted by

Mark A. Arnold

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2011

Master's Committee:

Advisor: Terry E. Engle

John J. Wagner
David C. Van Metre

ABSTRACT

EFFECTS OF COPPER CONCENTRATION AND SOURCE ON PERFORMANCE, BILE COMPONENTS, COPPER METABOLISM AND GASTROINTESTINAL MICROBIAL DISTRIBUTION IN NURSERY SWINE

One hundred twenty weaned nursery pigs (6.12 ± 0.56 kg) were utilized in this experiment to determine the effects of Cu concentration and source on performance, bile components, Cu metabolism, and gastrointestinal microbial distribution in nursery pigs blocked by weight and gender and placed in pens containing 5 pigs of similar weight distribution per pen. Pigs were fed one of four dietary treatments for 21 or 22d. Treatments consisted of: 1) Control (5 mg of Cu/kg from $CuSO_4$); 2) 250 mg of Cu/kg from $CuSO_4$, (250-sulfate) 3) 75 mg of Cu/kg from Cu-MINTREX®Cu (75-Min; Novus International, Inc., St. Charles, MO), and 4) 75 mg of Cu/kg from $CuSO_4$ (75-sulfate). On d 22 and 23, equal numbers of pigs per treatment were slaughtered. Post slaughter, blood, liver, intestinal tissue and contents, and bile samples were obtained. Body weights, ADG, and ADFI were similar across treatments ($P > 0.20$). Feed efficiency was greater ($P < 0.05$) for pigs receiving 250-sulfate compared to controls (0.53 vs. 0.43 ± 0.03, respectively), and pigs fed 75-Min (0.47 ± 0.03) or 75-sulfate (0.44 ± 0.03) were

intermediate. Pigs receiving 250-sulfate had greater ($P < 0.05$) bile (7.05 vs. 2.06 ± 0.49; respectively) and liver (124.4 vs. 53.3. ± 24.6, respectively) Cu concentrations than controls. Bile components, intestinal bacterial populations, and small intestine gene expression profiles (Ctr-1, Atox-1, Cox-17, ATP7a, and ATP7b) associated with Cu absorption and homeostasis were similar across treatments ($P > 0.20$). Antimicrobial effects of bile (determined by measuring the diameter of the zone of inhibition; mm) tended ($P < 0.20$) to be higher for 250-sulfate and 75-Min treatments compared to controls. Data from this experiment indicated that Cu dose influenced pig performance, but dose or source did not influence measured bile components, intestinal bacterial populations, or intestinal gene expression profiles associated with Cu absorption.

Acknowledgements

This experience has certainly been one of the most challenging yet rewarding things that I think I have ever done in my life. Throughout the process I have certainly learned quite a bit about myself and have had the opportunity to work with some of the brightest people within their respective fields. There are many people that have helped me along the way and without their support I don't know how I would have ever got through graduate school.

First of all I would like to thank my major professor Dr. Terry Engle. I know there were times throughout this process where I most definitely tested his patience, but I honestly don't think there is another professor I would have enjoyed working with more. Terry you are by far one of the brightest people that I have ever been around, I have learned more from you the past two years than I could have ever imagined. You were very supportive of everything I was involved in and I thank you for allowing me to be involved in everything like I was. Thank you for all of your support and patience, but more importantly thank you for your friendship and being a mentor to me.

I would also like to thank Dr. John Wagner and Dr. David Van Metre for agreeing to be on my committee and all of their help and support along the way.

A special thanks is most definitely in store to all the graduate students who helped with my project. From all the people who helped me build pens and set up the

metabolism barn to the people who helped me feed and everyone who helped through the euthanasia process, if it wasn't for you guys I don't know how I would have ever accomplished this study. A special thanks to Jenny Schutz, I really don't know how I would have done everything if it wasn't for your guidance along the way. You are a great person and a dear friend and I am very appreciative of everything you have done.

Last but not least, I would like to thank my family Mark, Debora, and Danielle. You guys have been the most supportive and encouraging people in my life and I know I wouldn't be where I am today without you guys. I love you and thanks for everything.

TABLE OF CONTENTS

PAGE

Chapter I

LITERATURE REVIEW

Chemical Properties of Copper

Copper (Cu) is a trace element essential to life for many plants and animals, usually occurring as part of the prosthetic group of oxidizing enzymes. Copper is the 29th element in the periodic table and is in group IB. It has a reddish brown color and its symbol is Cu. Copper is known as a micro (trace) mineral. The distinction between macro and micro minerals or trace elements is based on the relative amounts required in the diet for normal life processes (Pond et al., 2005). The major elements are required in concentrations of greater than 100 mg Cu/g DM and are often expressed as a percentage of the diet, whereas trace elements are required less than 100 mg Cu/g Dm and are expressed as ppm or sometimes as ppb (McDowell, 1992). Copper has an atomic number of 29 and an atomic weight of 63.5 (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 a ductile and malleable metal, a good thermal conductor, and is an excellent electrical conductor (Dameron and Howe, 1998). The common oxidation states are the cuprous (Cu(I) $3d^{10}$) or the cupric (Cu(II) $3d^{9}$) forms, however there are two other oxidation states: metallic Cu (Cu^{0}) and trivalent ion (Cu^{3+}). The trivalent form of copper is found in only a few compounds and is a strong oxidizing agent (Cotton and

Wilkinson, 1989). The metallic form is very stable in dry air at low temperatures but undergoes a slower reaction in moist air. Because of this reaction, a hydrocarbonate (a hydrous carbonate, as malachite) or hydrosulfate (a salt formed by sulfuric acid with an alkaloid or other organic base) forms a greenish-grey amorphous film over the surface which protects the underlying metal from attack from air (Dameron and Howe, 1998). Cotton and Wilkinson (1989) stated that the metal is soluble in water, but can be dissolved in nitric acid and sulfuric acid as well as in basic solutions.

Dameron and Howe (1998) stated that the chemistry of the element, especially in biological systems, is profoundly affected by the electronic/oxidation state that Cu resides in. The exchange between oxidation states gives the element redox properties which may be of an essential or deleterious nature in biological systems (Dameron and Howe, 1998). Copper (II) is the most important oxidation state in natural and aqueous environments (Dameron and Howe, 1998). Copper (I) and Copper (II) however are the most common oxidation states. Copper (I) is quickly oxidized by any oxidizing reagent (anything that metabolizes water, i.e. cytochrome C) present and Copper (II) ions via oxygen bind to inorganic ligands (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, transportation, and distribution of Cu to body tissues (Dameron and Howe, 1998).

Metabolism of Copper

In most animal species Cu is poorly absorbed; the extent of absorption is influenced by its chemical form (Underwood, 1977). Typically no more than 5-10% of dietary Cu is absorbed by adult animals, however younger animals are able to absorb a greater percentage (15-30%) of dietary Cu (McDowell, 1992). One reason for this is that mothers transmit Cu to the fetus before birth then through the milk after birth, therefore younger animals have a high affinity for Cu. When food enters the stomach weak Cu complexes are dissociated at a low pH (Allen and Solomons, 1984; Dameron and Howe, 1998). After disassociation in the stomach, Cu can bind to amino acids or oligopeptides (Allen and Solomons, 1984). 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 (Gollan and Dellor, 1973).

Monogastric species of livestock absorb Cu more effectively than mature non ruminant species and depending on the species studied, Cu can be absorbed in all segments of the GI tract (McDowell, 1992). Absorption occurs primarily in the upper section of the small intestine, however it can also be absorbed in other parts of the GI tract, but not as effectively with a lower absorption amount when compared to the amount absorbed in the upper small intestine (Allen and Solomon, 1984; McDowell, 1992; Dameron and Howe, 1998).

Absorption across the intestinal mucosa can be obtained in a two-step process (Crampton et al., 1965). One absorption pathway for Cu is transportation across the mucosal membrane, then it can be transported across the cell. Copper appears to be absorbed by two mechanisms, one saturable and the other unsaturable, suggesting active

transport for saturable and simple diffusion for the unsaturable (Bronner and Yost, 1985; McDowell, 1992). Transfer across the mucosal barrier probably occurs by non-energy-dependent diffusion (Dameron and Howe, 1998). When Cu is transferred across the basolateral membrane it appears to be rate-limiting and is mediated by a saturable, energy-dependent mechanism with levels of Cu normally ingested (Dameron and Howe, 1998). With increased intake levels, additional diffusion or carrier-mediated systems in the basolateral membrane are more essential, and it appears likely that they are the sites where competition for absorption between Cu and other transition metal ions takes place (Linder, 1991; Dameron and Howe, 1998).

Copper absorption may be dependent on the amount of Cu in the diet and when dietary Cu concentrations are low absorption is increased (Strickland et al. 1972; Giltin et al., 1960; Dameron and Howe, 1998). The cystol protein enhances or blocks the transfer of Cu into the blood so this regulates the amount of Cu transferred in the blood stream (Allen and Solomons, 1984). Copper absorption may also be regulated or restricted by metallothionein, which are cysteine-rich, cytosolic proteins capable of binding several metal ions under certain physiological conditions (Allen and Solomons, 1984; Hamza and Gitlin, 1996).

Absorbed Cu becomes loosely bound to serum albumin and amino acids and thereby transported throughout the body where the liver is the major storage organ (McDowell, 1992). Copper in the blood is mainly bound to albumin, which has specific and non-specific sites for Cu (McArdle et al., 1999; Linder et al., 1999). Some of these amino acids are histidine (His) and transcuprien (Lau et al., 1974; Schmitt et al., 1983; McArdle et al., 1999). Other small amounts of Cu in the blood are bound in plasma to

amino acids (Anon, 1981). The other amino acid that binds Cu is transcuprien. Transcuprien has a higher affinity for Cu than albumin and it readily changes Cu(II) with albumin (Linder et al., 1999). The natural accumulation of Cu^{2+} proceeds via assimilation of the naturally occurring proteins, albumin and transcuprien in the blood (Smith, 2004). It is carried to the liver and internalized by hepatocytes through the mediation of small amino acid complexes (Smith, 2004). Copper is sequestered through metallothioein in the form of Cu^{+} and Cu^{2+} ions. It is prepared for bilary excretion or utilized for incorporation into ceruloplasmin or other metalloenzymes (Smith, 2004). Ceruloplasmin is synthesized into hepatocytes and secreted into the plasma, it is then exported through the blood to other organs where it is induced to release Cu^{2+} ions (Smith, 2004).

Copper is carried throughout the blood stream and is primarily taken up by the liver and kidney once it is bound to these compounds (Linder et al., 1999). The liver is a very important organ for Cu homeostasis. The liver is the main storage and distribution organ for Cu (Dameron and Howe, 1998). Uptake of Cu across the hepatic membrane is believed to be carrier mediated and is not dependent on cell energy or coupled to the sodium (Na) gradient (McArdle et al., 1999). In the liver, hepatocytes are responsible for the uptake and storage of Cu, as well as the regulation and excretion of this metal into the bile (Hamza and Gitlin, 1996). Several pathways have been proposed to explain the transport of Cu into bile (Dameron and Howe, 1998). There are two transcellular pathways that exist through hepatocytes (Dameron and Howe, 1998). Copper transported into the bile takes place with bilary excretion of glutathione (Dameron and Howe, 1998). Glutathione is involved in the last step of Cu excreted from the hepatocyte into the bile

(Dameron and Howe, 1998). The hepatocytes remove Cu from the affinity sites on albumin (McArdle et al., 1990). A transporter brings the Cu into the hepatic cell membrane, the transporter is attached to two disulfide bridges (McArdle et al., 1999). One of the bridges is on the outside of the hepatic membrane, the second is on the inside of the membrane, this transmembrane protein hCTR1, transports Cu form the circulatory system into hepatocytes (McArdle et al., 1999). Little is actually 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 (McArdle et al., 1999). Copper (I) enters the bridge and is reoxidized, however, it is not known whether Cu(I) is reoxidized to Cu(II) before or after Cu enters the Golgi apparatus in the hepatic cell (McArdle et al., 1999). Copper is transported across the Golgi apparatus by ATP7B, which is energy dependent (McArdle et al., 1999). ATP7B is a Cu transporting P-type ATPase expressed within the secretory pathway of hepatocytes (Hamza and Gitlin, 1996).

There are numerous pools that Cu is divided into within the liver. These pools are used for transit, storage, and excretion (Owen, 1980). Hamza and Gitlin (1996) reported that under normal conditions the amount of Cu excreted in the bile will be directly proportional to the size of the pool. In the transit pool, Cu is incorporated into ceruloplasm and other Cu dependent enzymes such as Cu-Zn superoxide dismutase (SOD). Ceruloplasm is the carrier for the tissue specific export of Cu from the liver to the target organs (McDowell, 1992). Around ninety percent of Cu in plasma is bound to ceruloplasm with the rest being bound mainly to albumin, but transcuprein and ligands as well (McArdle et al., 1999).

Since the liver is the major storage organ for Cu, the Cu that is placed in the storage pool is stored in the liver as mitochondrial cuprein until it is needed (McDowell, 1992). Copper is also present in the cystol bound to a metallothionein-like protein (McDowell, 1992). The liver is capable of storing approximately twenty percent of the body's Cu supply. Copper in the liver can be mobilized and shipped to extrahepatic tissues when the body is in negative Cu balance (Dameron and Howe, 1998). Metallothionine may be essential or may play a role in the storage of Cu (McArdle et al., 1999). Metallothionine has the capability to bind up to twelve Cu(I) atoms, this aids in Cu storage and helps protect the body from Cu toxicities (Bremner, 1987). If the body is in a negative Cu balance it can move Cu stores from the storage pool to the transit pool to be utilized by the body if necessary.

The excretion pool is utilized to send Cu out of the body. In all species, a large amount of ingested Cu appears to be excreted through the feces (McDowell, 1992). However a large portion of this is unabsorbed Cu, and the most active excretion also occurs through the bile (McDowell, 1992; Underwood, 1977). Eighty percent of Cu excreted through the body is excreted through the bile (Winge and Mehra, 1990). The remainder of the Cu is excreted in smaller quantities through urine, milk, intestine, and small amounts are excreted in perspiration (McDowell, 1992; Underwood, 1977).

Physiological functions of Cu

Copper is an important mineral that is required to sustain normal function of the body. Copper plays an active role in a number of enzymes (Dameron and Howe, 1998). Copper has many functions throughout the body such as iron metabolism, cellular respiration, cross-linking of connective tissue, pigmentation and keratinization of hair and

wool, central nervous system development, reproduction, immunity, and lipid metabolism. Copper is also utilized by some structural proteins as well. Copper is an essential component of several physiologically important metalloenzymes, including cytochrome oxidase, lysyl oxidase, Cu-Zn dependent superoxide dismutase, dopamine-B-hydroxylase, and tyrosinase (McDowell, 1992).

Copper plays an essential role in iron (Fe) homeostasis (McDowell, 1992). Ceruloplasmin, which is synthesized in the liver and contains Cu, is necessary for the oxidation of Fe, permitting it to bind with the Fe-transport protein, transferrin (McDowell, 1992). Iron must be converted to the ferrous form to be mobilized from stored ferritin and incorporated into hemoglobin and myoglobin (McDowell, 1992). Furthermore, ceruloplasmin performs a reaction converting Fe to the ferric form, allowing Fe to be stored and/or incorporated into hemoglobin and myoglobin (McDowell, 1992).

Copper is also essential in cellular respiration. Copper is a component of cytrochrome C oxidase (Allen and Solomons, 1984; Dameron and Howe, 1998). This enzyme is the terminal oxidase in the respiratory chain and catalyzes the reduction of O_2 to water, an essential step in cellular respiration (McDowell, 1992).

Copper is utilized in the cross-linking of connective tissue. Lysyl oxidase is the key Cu-containing enzyme in the formation of the cross-links in collagen and elastin (McDowell, 1992). Both 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). These cross-links give the proteins structural rigidity and elasticity (McDowell, 1992).

Achromotrichia (lack of pigmentation) is a principal manifestation of Cu deficiency in many species (McDowell, 1992). Therefore, Cu plays an integral role in the development of pigmentation and keratinization of hair and wool. Tyrosinase (polyphenyl oxidase) is an enzyme that catalyzes the conversion of tyrosine to melanin the component responsible for pigmentation of hair and wool (McDowell, 1992). Copper is also required for the formation or incorporation of disulfide groups in keratin synthesis (McDowell, 1992). These disulfide groups provide cross-linkages or bonding of keratin and on alignment or orientation of long-chain keratin fibrillae in the fiber (McDowell, 1992).

The central nervous system is very dependent on Cu. Cytochrome C oxidase is an important enzyme primarily because this enzyme is essential for myelin formation. Myelin is composed largely of phospholipid (McDowell, 1992). The inhibition of myelin synthesis results in the ensuing neurological disturbance (McDowell, 1992). 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 plays a role in lipid metabolism, reproduction, and immunity. However, these subjects are beyond the scope of this literature review and will not be discussed.

Copper Deficiency and Toxicity

There are many factors that can possibly influence or onset a Cu deficiency and toxicity. Some of these factors are how the animal excretes the Cu, the availability of the mineral, and the chemical form of Cu. Each species of livestock will vary on how they will be affected by these factors. Sheep are affected the most but species such as swine

and cattle are also susceptible (Gupta and Gupta, 1998). The reason sheep are more susceptible to toxicities when compared to pigs and cattle is primarily because they cannot excrete Cu in bile as effectively as the other two species (Dameron and Howe, 1999). The main manifestation of a Cu deficiency includes anemia, diarrhea, bone disorders, reproductive failure, nerve disorders, cardiovascular disorders, achromotrichia (loss of hair pigment), and keratinization failure in hair and wool (McDowell, 1992). Along with these manifestations, a decrease in appetite occurs in all species. There are other factors that can possibly cause deficiencies, one of which is the chemical form of copper. Knowing there are different forms of copper available, certain animals may utilize one form different then they do with another. Copper carbonate ($CuCO_3$) is more available than copper sulfate ($CuSO_4$) and $CuSO_4$ is more available than Cu oxide (CuO) (Allen and Solomons, 1984). Therefore, if copper oxide were the only copper source available to swine, they may become copper deficient because copper oxide is poorly available to swine (Cromwell et al., 1989).

Copper deficiencies and toxicities can occur in all species; however the focus of this next section is primarily on swine. Although, few naturally occurring deficiencies have been observed in pigs on typical diets (Miller et al., 1979), they are still likely to have disorders with a deficiency. An early sign of Cu deficiency in the pig is a microcytic and hypochromic anemia (McDowell, 1992). Teague and Carpenter (1951) comprised early studies of Cu deficiencies in pigs and noted a decrease in corpuscular volume and hemoglobin concentrations. Alongside with anemia, an unusual leg condition develops in Cu-deficient pigs (Teague and Carpenter, 1951; McDowell, 1992). The animal is very unstable in its rear leg joints and has an excessive set to the hock; this

makes the animal carry its legs underneath it and forces it to a sitting position. There are various degrees of crookedness through the front legs and forelimbs (McDowell, 1992). There is also a failure of bone deposition (McDowell, 1992). Miller et al. (1979) noted cardiovascular and central nervous system disorders in Cu deficient swine. The heart increases 200% in size and ruptures major blood vessels, suggesting a problem with elastin tissue (McDowell, 1992). Another problem with swine has been decreased rate of growth with a severe copper deficiency.

Cu Deficiencies

Copper deficiency is associated with a decline of the element in liver, kidney, rib, brain, and other tissues (McDowell, 1992). There are many problems that occur during a Cu deficiency. Anemia is a problem that can occur as a result of a Cu deficiency (McDowell, 1992). With anemia ceruloplasm production is decreased in the liver because of an impairment of Fe mobilization (Baker and Ammerman, 1995; Owen, 1965; Harris and Disilvestro, 1981).

A deficiency of Cu can cause changes and disorders in bones and joints. In Cu deprived animals there is a reduction in the activity of lysyl oxidase which could possibly diminish the stability and strength of bone collagen and impair mineralization of the cartilage with reductions in bone strength (Underwood and Suttle, 1999; Opsahl et al., 1982). Lysyl oxidase is important in the conversion of lysine to desmosine, which is important in the cross-linking of collagen (Committee on Animal Nutrition et al., 1980; Underwood and Suttle, 1999). When the collagen is unable to undergo cross-linking, cartilage and strength of the bone are compromised.

Cardiovascular disorders can also occur with Cu deficiency and have been discovered in all species. Anatomical studies of several species of Cu deficient animals revealed, among other things, aortic fissures and rapture, arterial foam cells and smooth muscle migration, cardiac enlargement and rupture, coronary artery thrombosis and myocardial infractions (Klevay, 2000). There is a corresponding alteration in mechanical properties of affected aortas that is a reflection of altered mechanical properties of the elastin (Coulson and Carnes, 1967). Copper is important for elastin synthesis (Underwood and Suttle, 1999). The elastin is utilized to contract and expand the arteries and veins without tearing. Problems occur when the arteries or veins are not able to contract. Aortic ruptures have occurred during Cu deficiencies because of reduced elasticity (Underwood and Suttle, 1999; Dameron and Howe, 1998).

Copper deficiencies also cause decreased Cu concentrations in the heart and other organs and cells and increases cholesterol in plasma (Klevay, 2000). Copper depletion experiments have also revealed abnormalities of lipid metabolism, blood pressure control, and impaired glucose tolerance (Klevay, 2000).

The immune system can also be affected by a deficiency. Some immune functions such as T-cell, B-cell, neutrophils, and macrophage production have decreased due to a deficiency (McDowell, 1992).

There have been deficiency studies that result from other antagonistic minerals. Some of these minerals are Iron (Fe), Zinc (Zn), Cadmium (Cd), Silver (Ag), and Lead (Pb). At times when these minerals are fed at higher levels in the diet it can cause a Cu deficiency. Also if these minerals are low in the diet and Cu is high it can also cause

toxicity. Many of these minerals are dependent upon each other and can affect the body by a deficiency/toxicity if not in the diet at the right amounts.

Cu Toxicity

Copper poisoning, acute or chronic, is encountered in most parts of the world (McDowell, 1992). Acute poising is usually observed after accidental administration of excessive amounts of Cu salts which may be present in anthelminthic drenches, mineral mixes and improperly formulated rations (Gupta and Gupta, 1998). There are factors such as low levels of Mo or sulfate in the diet that enhance the absorption or retention of Cu, therefore resulting in a Cu toxicity or poising (Gupta and Gupta, 1998). Ruminants are more sensitive to Cu toxicity than nonruminants, primarily because nonruminants have relatively high tolerance for Cu (McDowell, 1992). However when swine are fed higher Cu diets (250 mg Cu/kg DM), other nutrients could be adversely affected, such as a destruction of natural feed tocopherols (McDowell, 1992). Sheep are affected the most but species such as swine and cattle are also susceptible (Gupta and Gupta, 1998). Ruminants are most sensitive to toxicity, where a concentration in a cattle diet would be around 100mg Cu/kg DM with a tolerance between 100 and 400 mg Cu/kg DM (McDowell, 1992). With sheep being the most susceptible, toxicity levels in sheep are around 40 to 50 mg Cu/kg in the diet (NRC, 1985). In ruminants, if concentrations of Cu in the liver reach or exceed 1000 mg Cu/kg of DM, Cu can be released in the blood and Cu concentrations can be 10 times that of normal values. This results in a hemolytic crisis and could result in death (Underwood, 1981).

There are preventive measures to prevent Cu toxicity. Maintaining a ration and keeping the proper ratios of minerals in the ration is very important. In ruminants, to

treat toxicity both Mo and S should be administered (McDowell, 1992). Feeding higher amounts of zinc (Zn) in the diet could also be a preventive measure. For monogastric animals, Zn is more protective against Cu toxicity than is a combination of Mo and S. A higher amount of Zn in the diet reduces liver Cu storage (Pope, 1971). The addition of more Zn and Fe in the diet has helped prevent harmful effects from using 250 ppm Cu in the diet (Cunha, 1977).

Organic vs. Inorganic Sources of Copper

Bioavailability is defined as a proportion of an ingested element that is absorbed and then transported to its specific cite of action (Ledoux et al., 1991). There are different functions of bioavailability and utilization consists of absorption, transport to the site of function, and transformation metabolically to a biological form. Zinc and Cu are widely available as feed additives in two main forms, organic and inorganic (Hellman and Carlson, 2003). An "organic" feed source means that the mineral is bound to an organic material (Hellman and Carlson, 2003). Baker and Ammerman (1995) reported that the relative biovailability (a measurement of a drug when compared with another formulation of the same drug) estimates of organic Cu sources ranged between 88% and 147% of the response to cupric sulfate in poultry, swine, sheep, and cattle. Some of these materials are amino acid complexes, proteinates, chelates, polysaccharide complexes, and propionates (Hellman and Carlson, 2003). The organic form of a mineral may actually prevent binding of antagonists and other molecules, it does this because they are already bound leaving no available sites to bind to. The trace mineral in an inorganic supplement has been combined with an inorganic salt, such as zinc carbonate, zinc sulfate and zinc chloride (Hellman and Carlson, 2003). Animals being fed inorganic sources of minerals

have to convert them to the active forms before the body can utilize the mineral (Spears, 1996). Copper traditionally has been supplemented in inorganic forms. Some of the inorganic sources of Cu are Cu sulfate ($CuSO_4$), Cu chloride ($CuCl_2$), cupric oxide (Cu_2O), Cu oxide (CuO), and Cu carbonate ($CuCO_3$). Cu sulfate is the inorganic form of Cu used the most in animal diets.

Although both organic and inorganic forms are in common use, there are still important differences that exist in the bioavailability concerns associated with the two different forms. There have been debates over which source offers more bioavailability to the animal. The bioavailability of a mineral is determined by its interaction with other minerals (Hellman and Carlson, 2003). The more bioavailable a mineral becomes, the lower the dietary concentration needed to attain a given level of absorption and less mineral excreted to the environment (Hellman and Carlson, 2003). Spears (1996) stated there is a theory that organic sources of minerals are more similar to the active forms of minerals in the body and in feed.

In recent years there has been more of an increase in feeding the organic form of Cu, primarily because of improved performance/growth, reproductive efficiency, and health. Organic forms of Cu are bound to amino acids or protein in different types of minerals marketed as complexes, chelates, or proteinates (Spears, 1996). The complexes are formed when a ligand binds to a metal ion. Chelation refers to a special type of complex formed between a ligand and a metal ion (Spears, 1996). In a chelated form, metals are absorbed more efficiently and their retention in the body is higher than similar minerals in their inorganic form (Fouad, 1976). Proteinates are metals that are chelated to amino acids and are partially hydrolyzed proteins.

Performance, Cu status, and Gastrointestinal Microfilaria

Feeding pharmacological concentrations of Cu (125 to 250 mg of copper/kg diet DM) to growing pigs improves rate of gain and efficiency of growth (Braude, 1967; Wallace, 1968). However, other studies have indicated no direct results among treatment groups where average daily gain and performance was not affected by Cu source through the nursery period (Stansbury et al., 1990; Bradley et al., 1983).

Barber et al. (1955 found a significant growth-promoting effect by adding $CuSO_4$ to a growing-pig diet, since then, this has been widely confirmed. Barber et al (1960) conducted an experiment to determine $CuSo_4$ and CuS as supplements for growing pigs. Pigs (n = 72) were utilized in two experiments. Barber et al. (1960) concluded that a supplement of 250 ppm Cu given as $CuSo_4$ had no significant effect on performance in the first experiment, but in the second it significantly increased growth rate by 12.9% and food consumption by 8.8%. Copper Sulfate, not CuS, in the diet significantly increased liver and kidney stores of Cu.

In 1983, Bradley et al conducted an experiment to determine the effects of graded levels of dietary Cu on Cu and Fe concentrations in swine tissues. Weanling pigs (n = 120) were utilized in the study with six different treatment groups ranging from 7.5 to 240 ppm Cu in the diet. Average daily gain was not affected by dietary treatments and had no effect on performance; however, dietary Cu increased liver and kidney Cu. In 1990, Stansbury et al. conducted four experiments to determine the effect of Cu source and level and an antimicrobial agent on performance of nursery and growing pigs. In the study copper was fed either as $CuSO_4$ $5H_2O$ (CS), inorganic chelated Cu (ICC), or organic chelated Cu (OCC). In the first experiment, 224 pigs were used to study Cu

source and level added to nursery diets. Stansbury et al. (1990) reported no difference among treatments was observed during the nursery period. However, regardless the Cu source, pigs receiving 125 ppm added Cu gained faster than pigs in other treatments. In the second experiment, 216 pigs were used to determine the optimum level of CS and ICC in nursery diets. Stansbury et al. (1990) reported that pigs were less efficient when Cu was added at 62.5 and 125 mg Cu/kg DM than at 250 mg Cu/kg DM. In experiments three and four, Stansbury et al. (1990) reported no differences between OCC and CS on performance and pigs receiving ICC in their diets were less efficient than pigs on other treatments.

The growth-stimulation action of dietary Cu has been attributed to its antimicrobial actions; i.e. Cu inhibits the growth of enteric disease causing microorganisms, thus eliciting an antibiotic-like effect (Fuller et al., 1960). In 2004, Hojberg et al conducted an experiment to determine the influence of dietary zinc oxide and $CuSO_4$ on the GI ecosystem in newly weaned piglets. In the experiment 32 pigs were separated into 4 groups of 8 and fed four different treatment groups; (i) low Zn, low Cu (ii) low Zn, high Cu (iii) high Zn, low Cu (iv) high Zn, high Cu. The gastrointestinal tracts were immediately removed from the body cavities of the slaughtered piglets and divided into 8 sections. Hojberg et al. (2004) concluded that feed intake and daily gain were numerically lower for piglets fed the high level of dietary Cu than those fed the low levels. The addition of $CuSo_4$ had no significant influence on the digesta content. No differences in pH were observed after the addition of $CuSo_4$ to the feed. For the microbial populations the addition of $CuSO_4$ to the feed had no significant effect on the total counts of anaerobes in any of the gut segments.

Summary

Copper is part of many physiological functions in the body and is incorporated into many enzymes. Some of the functions are Fe metabolism, elastin and collagen formation, and immunity. Without Cu in the diet, a Cu deficiency would occur. Copper deficiency can cause bone disorders, cardiovascular disorders, achromotrichia, and reproductive failures. Higher amounts of dietary Cu can cause Cu toxicity that could possibly lead to death. There have been many studies conducted testing the effects of Cu on the body under different conditions. There have been studies conducted to focus on inorganic vs. organic sources of Cu and Cu source on performance and which sources are more available to the animal. Although performance and feed efficiencies have increased with higher amounts of dietary Cu, results have been inconsistent with inorganic vs. organic sources. There has not been enough research compiled on the microbial effects of dietary Cu in the gut, therefore more research is needed to determine the effects of Cu on the body and the microbial effects in the GI tract.

Chapter II

EFFECTS OF COPPER CONCENTRATION AND SOURCE ON PERFORMANCE, BILE COMPONENTS, COPPER HOMEOSTASIS AND GASTROINTESTINAL MICROBIAL DISTRIBUTION IN NURSERY SWINE

ABSTRACT: One hundred twenty weaned nursery pigs (6.12 ± 0.56 kg) were utilized in this experiment to determine the effects of Cu concentration and source on performance, bile components, Cu metabolism, and gastrointestinal (GI) microbial distribution in nursery pigs blocked by weight and gender and placed in pens containing 5 pigs of similar weight distribution. Pigs were fed one of four dietary treatments for 21 or 22 d. Treatments consisted of: 1) Control (5 mg of Cu/kg from $CuSO_4$); 2) 250 mg of Cu/kg from $CuSO_4$, (250-sulfate) 3) 75 mg of Cu/kg from Cu-MINTREX®Cu (75-Min; Novus International, Inc., St. Charles, MO), and 4) 75 mg of Cu/kg from $CuSO_4$ (75-sulfate). On d 22 and 23, equal numbers of pigs per treatment were slaughtered. Post slaughter, blood, liver, intestinal tissue and contents, and bile samples were obtained. Body weights, ADG, and ADFI were similar across treatments ($P > 0.20$). Feed efficiency was greater ($P < 0.05$) for pigs receiving 250-sulfate compared to controls (0.53 vs. 0.43 ± 0.03, respectively), and pigs fed 75-Min (0.47 ± 0.03) or 75-sulfate (0.44 ± 0.03) were intermediate. Pigs receiving 250-sulfate had greater ($P < 0.05$) bile (7.05 vs. 2.06 ± 0.49; respectively) and liver (124.4 vs. 53.3. ± 24.6; respectively) Cu

concentrations than controls. Bile components, intestinal bacterial populations, and small intestine gene expression profiles (Ctr-1, Atox-1, Cox-17, ATP7a, and ATP7b) associated with Cu absorption and homeostasis were similar across treatments ($P > 0.20$). Antimicrobial effects of bile (determined by measuring the diameter of the zone of inhibition; mm) tended ($P < 0.20$) to be higher for 250-sulfate and 75-Min treatments compared to controls. Data from this experiment indicated that Cu dose influenced pig performance, but dose or source did not influence measured bile components, intestinal bacterial populations, or intestinal gene expression profiles associated with Cu absorption.

Key words: copper, intestinal microflora, nursery pigs,

Introduction

Feeding pharmacological concentrations of copper (Cu; 125 to 250 mg of Cu/kg diet DM) to growing pigs improves rate of gain and efficiency of growth (Braude, 1967; Walace, 1968). However, the mechanisms by which Cu elicits these growth effects have not been elucidated. The growth-stimulation action of dietary Cu has been attributed to its antimicrobial actions; i.e. Cu inhibits the growth of enteric disease causing microorganisms, thus eliciting an antibiotic-like effect (Fuller et al., 1960). Furthermore, intravenously injected Cu has been shown to stimulate the growth of weaning pigs (Zhou et al., 1994). There are two possible ways that injected Cu could affect growth. One is that injected Cu may be released into the GI tract through the bile and act enterically (Zhou et al., 1994). The other possibility is that systemic growth-promoting actions of Cu could cause a possible enteric growth promoting action of Cu in pigs fed Cu (Zhou et

al., 1994). This suggests a systemic mode of action that could possibly complement the antimicrobial hypothesis proposed by Fuller et al. (1960). However, no definitive data are present to explain the impact of Cu on growth performance in pigs. Therefore, the objective of the present study was to determine the effects of Cu concentration and source on performance, bile components, and gastrointestinal microbial distribution in newly weaned pigs.

Materials and Methods

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

One hundred twenty nursery pigs (13.46 ± 1.23 lbs) were utilized in this experiment. Nursery pigs were obtained from a commercial swine operation 2 d post weaning and transported to Colorado State University's Agriculture Research, Development and Education Center (ARDEC) in Fort Collins, CO. Upon arrival, all pigs were housed in an environmentally controlled metabolism barn in groups of 5 pigs/pen and fed a basic ground corn based diet (Phase 1; Table 1) for 5 d. After the 5 d adaptation period, all pigs were weighed on d -1 and given an ear tag that contained the individual animal number and treatment. Pigs were then reweighed on d 0 of the experiment, blocked by weight and gender, and placed in pens containing 5 pigs per pen. Pens (3m x 3.5m) were equipped with automatic waterers and self feeders (n = 24 pens). Pigs were housed in a thermo-controlled room with adequate ventilation

Growing phase: After pigs were blocked and randomized into pens, they were transitioned from phase 1 of the feeding program to phase 2 (Table 2). During the second

phase of the feeding program, pigs were fed one of 4 Cu treatments for 21 d. Treatments consisted of: 1) Basal diet with NRC (1996) concentrations of Cu (**Control**; 5 mg of Cu/kg DM from Cu SO_4); 2) Control + 250 mg of Cu/kg DM from $CuSO_4$, (**250-$CuSO_4$**); 3) Control + 75 mg of Cu/kg DM from Cu-Mintrex (**75-Mintrex**); and 4) Control + 75 mg of Cu/kg DM from $CuSO_4$ (**75-$CuSO_4$**). Experimental diets were fed as mash diets and a common basal diet was formulated and mixed. Each treatment diet was prepared by the addition of a carrier (ground corn) with the appropriate concentration and source of Cu added. Individual health status and room temperature was recorded daily along with feed intake for each pen. Pigs were weighed during the middle of trial (d10) and at the end of trial (d 21). On d 22 and 23, equal numbers of pens per treatment were slaughtered via electrical stunning followed by exsanguination. Post slaughter, blood, liver, intestinal tissue and contents, and bile samples were obtained.

Tissue collection and analytical procedures:

Blood: Post slaughter, 6 ml of blood was obtained from the exposed jugular vein with a syringe. Three ml of blood was placed in vacutainer tube that contained no additive and three ml of blood were place in a vacutainer tube that contained sodium citrate (Becton Dickenson Co., Franklin Lakes, NJ). Blood was stored on ice, transported to the laboratory and stored at 5°C for 12 h. Blood was then centrifuged at 1200 x *g* for 25 minutes at room temperature. The serum and plasma were transferred to separate polyethylene tubes (12 mm X 75 mm) and stored at -70°C until analyzed.

Liver: Liver in its entirety was weighed, placed in an individual bags, stored on ice, and transported to the laboratory and stored ay -20°C until analyzed. Upon thawing, a 2.54 cm x 2.54 cm sub-sample from each liver was removed from approximately the

same anatomical location and combined with the other liver sub-samples within a pen of animals and minced with scissors until homogenous. Liver Cu concentration was determined via flame atomic absorption spectrophotometry (Varian Model 1275; Walnut Creek, CA). Briefly, liver tissue samples were dried at 80^{o}C for 24 h. Samples were weighed, placed into crucibles, and then dry ashed at 600^{o}C for 12 h. Two milliliters of 3.6 N 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 deionized H_2O to fit within a linear range of a standard curve.

Bile: Total bile volume was determined by aspirating all bile from the gall bladder. Then a subsample of 8-10 ml of bile per pig combined in a 50 ml conical tube by pen. The sample was stored on ice, transported to the laboratory and stored in a refrigerator at 5°C. Bile samples were then analyzed for Cu concentrations via flame atomic absorption spectrophotometry (Varian Model 1275; Walnut Creek, CA). Briefly, one ml of 10% trichloroacetic acid was added to one ml of plasma or standard and was mixed vigorously. The mixture was placed in a -20^{o}C freezer for 30 minutes to aid in precipitation and then centrifuged at 1200 x *g* for 10 minutes. The supernatant was removed, placed into a clean tube and then diluted in de-ionized H_20 to fit within a linear range of a standard curve generated by linear regression of known Cu concentrations. Bile samples were then used in a separate assay to examine the influence of bile on microbial growth. Briefly, 5 different species of organisms isolated from the control pig intestines: *Salmonella*, *E. coli*, *Citrobacter*, *Enterobacter*, and *Campylobacter* were utilized. Lawns of *Salmonella, E. coli, Citrobacter, and Enterobacter* were prepared on Trypticase Soy Agar (TSA) plates (Becton, Dickinson and Company; LePont de Claix,

France). Wells were punched in the agar with the back side of a sterile Pasteur pipette. Bile was diluted ten-fold from 10^{-1} to 10^{-5} in Trypticase Soy Broth (TSB; Neogen; Lansing, Michigan) and 25 µL of each dilution was added to each well in duplicate. After incubation at 37 °C for 36 h, zones of inhibition were measured. The same procedure was used for the *Campylobacter*, however the bacteria was grown on CCDA plates (Neogen; Lansing, Michigan) under aerobic conditions in an incubator. Bacteria was grown under aerobic conditions in the incubator to simulate the partial aerobic conditions that would exist inside the intestines.

To determine the composition of gall bladder bile (pooled within pen) was extracted with 10 vol. of ethanol at room temp, and the solvent was evaporated to dryness as described by Kibe et al. (1980) and Tangerman et al. (1986). Briefly, the solvent system for TLC were chloroform/ methanol/acetic acid/water (65: 20:10:5,v/v), n-butanol/acetic acid/water (85:10:5, v/v) and benzene/isopropanol/acetic acid (30:10: 1,v/v;). Quantitation of individual bile acids was carried out by GLC after hydrolysis, and methylation (Kibe et. al., 1980; Murata et al., 1978) and Tangerman et al. (1986).

Intestinal Bacteria Sampling: The small (early and mid jejunum) and large intestines (20 cm from the ileocecal juncture) were separated and as much of the contents of each segment (large and small intestine) as we could obtain were then collected separately for the large and small intestine and placed into a sterile Whirlpack bag for each pen. Samples were immediately transported to the laboratory and cultured for *Salmonella, E. coli, Enterobacter, Lactobacillus,* and Coliform bacteria.

Salmonella: Five mg of sample from the large and small intestine were placed into 45 ml of sterile buffered peptone water (Neogen; Lansing, Michigan) and incubated

at 35° C for 24 h. Aliquots were then transferred to two different types of sterile selection broth, Rappaport-Vassiliadis Broth (RVS; Neogen; Lansing, Michigan) and Tetrathionate Broth (Becton, Dickinson and Company; Le Pont de Claix, France). Samples were incubated at 42° C for 24 h and then streaked for isolation onto Hektoen-Enteric (HE) and XLT4 plates (Neogen; Lansing, Michigan) and incubated at 37° C for 24 h. The appropriate colonies were removed from the plate and streaked onto a CHROM Salmonella Agar (DifCo; Becton, Dickinson and Company; Le Pont de Claix, France). The colonies were also inoculated onto the Triple Sugar Iron Agar slants and the Lysine Iron Agar slants. Presumptive salmonella isolates were sent to the Colorado State University Diagnostic Lab for evaluation.

Campylobacter: A 1.0 g sample from the large and small intestine were placed into 13 mL of Bolton broth (Oxoid LTD; Basingstoke, Hampshire, England) and incubated under microaerobic conditions generated in gas jars with BD Gas Pack EZ Container Campy Container System Sachets for 4 h at 42° C. Cultures were further incubated for 44 h at 42° C and then streaked onto Charcoal-Cefoperazone-Deoxycholate Agar (CCDA) plates (Neogen; Lansing, Michigan). Plates were cultured under aerobic conditions for 3-5 d at 42° C. *Campylobacter* was confirmed by agglutination with a Camylobacter Latex Kit (Hardy Diagnostics, Santa Maria, California) and then used in the bile assay.

E. coli, Lactobacillus, and Coliforms: A 1.0 g sample from the large and small intestine contents was placed in 9 mL of 0.1% buffered peptone water. Ten-fold serial dilutions were prepared and the $1x10^{-4}$, $1x10^{-6}$, and $1x10^{-8}$ dilutions were plated onto E. coli and Coliform Petrifilms. Petrifilms were incubated for 48 h, and the appropriate

colonies were counted. For the *Lactobacillus* counts, 1 mL of the appropriate dilution was mixed into 9 ml deMan-Rogosa-Sharpe (MRS) molten agar, poured into plates, allowed to harden, and incubated for 48 h at 37° C. Pour plates were used to create anaerobic conditions. After the 48 h incubation, the colonies were counted.

Statistical Analysis: Statistical analysis of data was performed using a mixed model procedures as described by SAS (release 9.2, SAS Institute, Cary, NC). The model included the fixed effect of treatment. Random effects were pen within treatment. Treatment and treatment x period interactions were considered to be significant if $P < 0.05$.

Results and Discussions

Performance

Previous studies have shown that feeding pharmacological concentrations of Cu (125 to 250 mg of copper/kg diet DM) to growing pigs improves rate of gain and efficiency of growth (Braude, 1967; Wallace, 1968). However, other studies have indicated no direct results among treatment groups where ADG and performance was not affected by Cu source through the nursery period (Stansbury et al. 1990; Bradley et al., 1983). In this study body weights, ADG, and DMI were similar across all treatments ($P > 0.05$). However, FE was significantly improved ($P < 0.02$) for pigs supplemented with 250 mg of $CuSO_4$ / kg DM when compared to the control. Similar results were found in a study conducted by Stansbury et al. (1990), where 231 nursery pigs allotted to 7dietary treatment groups based on source and level of copper (source = inorganic chelated or organic chelated at 62.5, 125, or 250 ppm) revealed no significant effects ($P > 0.001$) for

IW, FW, or ADG. However, in contrast to this study, no significant results were found for ADFI or FE as well. Feed efficiency was significantly greater ($P < 0.02$) for pigs supplemented with 250 ppm $CuSO_4$; this is similar to previous studies conducted by Barber et al. 1960, where rate of gain and food consumption from pigs receiving $CuSO_4$ was significantly greater than their contemporary groups given CuS at either level.

Cu Status on Tissues

Copper status for bile and liver samples were significantly greater ($P < 0.05$) for pigs supplemented with 250 mg $CuSO_4$/kg DM and treatment groups being fed Cu when compared to the control group. This is consistent with previous studies conducted by Bradley et al. (1983) and Barber et al. (1960). Bradley et al. (1983) utilized 120 weanling pigs with six different treatment groups ranging from 7.5 to 240 ppm Cu to determine the effects of dietary Cu on performance and Cu stores in selected swine tissues. Bradley et al. (1983) reported that 120 and 140 ppm dietary Cu increased ($P < 0.01$) liver Cu and kidney Cu ($P < 0.05$). It can be hypothesized that feeding increased amounts of dietary Cu causes increased amounts of Cu in specific tissues.

Effects of Cu on Microbial Growth

The growth-stimulation action of dietary Cu has been attributed to its antimicrobial actions; i.e. copper inhibits the growth of enteric disease causing microorganisms, thus eliciting an antibiotic-like effect (Fuller et al., 1960). Increased amount of dietary Cu had no affect with amount of bacteria grown in the small and large intestines. Coliform, E. coli, Enterobacteriaceae, and Lactobacilus counts were similar across treatments ($P > 0.05$). Similar results were found in a study conducted by Hojberg et al. (2004), where 32 piglets allotted to 4different treatment groups based on source and

level of Cu and Zn. The study revealed that the addition of $CuSO_4$ to the feed had no significant effect on the total counts of anaerobes in any of the gut segments ($P > 0.22$).

Conclusions

These data indicate that feeding increased amounts of dietary Cu for 21 d to newly weaned pigs improves Fe and Cu status on specific tissues. Furthermore, increased amounts of dietary Cu had no impact on performance, specifically body weights, ADG, and DMI, and had no impact on microbial growth in the gastrointestinal tract of the pigs. Further studies are needed to gain a more fundamental understanding of the impact of copper source and bile composition on microbial growth in the gastrointestinal tract of newly weaned pigs.

Literature Cited

Allen, L.H. and N.W. Solomons. 1984. Copper. In: N.W. Solomons and Irwin H. Rosenberg (ed.). Absorption and malabsorption of mineral nutrients. Alan R. Liss, Inc, New York, NY. pp. 199-229.

Anon. 1981. Copper metabolism in premature and low-birth-weight neonates: Nutr. Rev. 39: 333-336.

Baker, D.H. and C.B. Ammerman. 1995. Copper bioavailability. In: C.B. Ammerman, D.H. Baker, and A.J. Lewis (Ed). Bioavailability of Nutrients for Animals: Amino Acids, Minerals, and Vitamins. Academic Press, San Diego, CA. pp. 127-156

Barber, R.S., R. Braude, K.G. Mitchell, and J. Cassidy. 1955. High copper mineral mixture for fattening pigs. Chem. Ind. (Lond.) 21:601.

Barber, R.S., J.P. Bowland, R. Braude, K.G. Mitchell, and J.W.G. Porter. 1960. Copper sulphate and copper sulphide (CuS) as supplements for growing pigs. Brit. J. Nutri. 15:189.

Bradley, B.D., G. Graber, R.J. Condon, and L.T. Frobish. 1983. Effects of graded levels of dietary copper on copper and iron concentrations in swine tissues. J. Anim. Sci. 56: 625.

Braude, R. 1967. Copper as a stimulant in pig feeding. World Rev. Anim. Prod. 3, p. 69

Bremner, I. 1987. Involvement of metallothionein in the hepatic metabolism of copper: Critical review. J. Nutr. 117: 19-29.

Bronner, F. and J.H. Yost. 1985. Saturable and non-saturable copper and calcium transport in mouse duodenum. Am. J. Physiol. 249: G108-112.

Committee of Animal Nutrition, Board on Agricultural and Renewable Resources, and Commission on Natural Resources, and NRC. 1980. National Academy of Sciences, National Academy Press, Washington, DC.

Cotton, F. A and G. Wilkinson. 1989. Advanced inorganic chemistry. John Wiley and Sons Ltd, New York, NY. pp. 755-775.

Coulson, W.F., and W.H. Carnes. 1967. Cardiovascular studies on copper deficient swine. IX. Repair of vascular defects in deficient swine treated with copper, Amer. J. Pathol. 50

Crampton, R.F., D.M. Matthews, and R. Poisner. 1965. Observation on the mechanism of absorption of copper by the small intestine. J. Physiol. 178: 111.

Cromwell, G.L., T.S. Stahly, and H.J. Monegue. 1989. Effects of source and level of copper on performance and liver copper stores in weanling pigs. J. Anim. Sci. 67: 2996.

Cunha, T.J. 1977. Swine Feeding and Nutrition. Academic Press, New York

Dameron, C. and P.D. Howe. 1998. Copper: Environmental Health Criteria 200 World Health Organization, Geneva.

Fouad, M.T. 1976. The physiochemical role of chelated minerals in maintaining optimal body biological functions. J. Appl. Nutr. 28:5.

Fuller, R., Newland, L.G.M., Briggs, C.A.E., Braude, R. and Mitchell, K.G. 1960. The normal intestinal flora of the pig. IV. The effect of dietary supplements of penicillin, chlortetracycline or copper sulphate on the faecal flora. Journal of Applied Bacteriology 23 (2), 195-205.

Gitlin, D., W.L. Hughes, and C.A. Janeway. 1960. Absorption and excretion of copper in mice. Nature. 188: 150-151.

Gollan, J.L and D.J. Dellor. 1973. Studies on the nature and excretion of biliary copper in man. Clin. Sci. 44: 9-15.

Gupta, U.C. and S.C. Gupta. 1998. Trace element toxicity relationships to crop production and livestock and human health: implications for management', Communications in soil science and plant analysis, 29: 11, 1491-1522

Hamza, I. and J.D. Gitlin. 1996. Hepatic copper transport. National Library of Medicine. Madame Curie Bioscience Database. Internet. Austin. TX

Harris, E.D., and R.A. DiSilvestro. 1981. Correlation of lysly oxidase activation with the p-phenylenediamine oxidase activity (ceruloplasmin) in serum (41102). Proc. Soc. Exp. Bio. Med. 166: 528-531.

Hellman, H. and M. Carlson. 2003. Feeding organic and inorganic sources of trace minerals for swine production. University of Missouri Extension publication G2323.

Hojberg, O., N. Canibe, H.D. Poulsen, M.S. Hedemann and B.B. Jensen. 2004. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Applied and Environmental Microbiology. p. 2267-2277.

Kibe, A., C. Wake, T. Kuramoto, and T. Hoshita. 1980. Effect of dietary taurine on bile acid metabolism in guinea pigs. Lipids. 15: 224-229.

Klevay, L.M. 2000. Trace element and mineral nutrition in disease: Ischemic heart disease. In Clinical Nutrition of the Essential Trace Elements and Minerals: The guide for health professionals. Human Press, Totowa, N.J.

Lau, S.Y., T.P.A. Kruck, and B. Sarker. 1974. Peptide molecule mimicking the copper (II) transport site of human serum albumin. J. Biol. Chem. 246: 5878-5884.

Ledoux, D.R., P.R. Henry, C.B. Ammerman, P.V. Rao, and R.D. Miles. 1991. Estimation of the relative bioavailability of inorganic copper sources for chicks using tissue uptake of copper. J. Anim. Sci. 69:215.

Lide, D.R. and H.P.R. Frederikse. 1993. CRC handbook of chemistry and physics, 74th ed. Boca Raton, FL, CRC. Press.

Linder, M.C. 1991. The biochemistry of Copper. Plenum Press, New York, NY.

Linder, M.C., N.A. Lomeli, S. Donley, F. Mehrbod, P. Cerveza, S. Cotton, and L. Wooten. 1999. Copper transport in mammals. In: A. Leone and J.F.B. Mercer (ed.) Copper transport and its disorders: molecular and cellular aspects. Kluwer Academic/Plenum Publishers, New York, NY. pp.1-16.

Marceau, N., N. Aspin, and A. Sass-Kortsak. 1970. Absorption of copper 64 from gastrointestinal tract of the rat. Am. J. Physiol. 218: 377-383.

McArdle, H.J., M.J. Bingham, K. Summer, and T.J. Ong. 1999. Cu metabolism in the liver. In: A. Leone and J.F.B. Mercer (ed.) Copper transport and its disorders: molecular and cellular aspects. Kluwer Academic/Plenum Publishers, New York, NY. pp. 29-37.

McArdle, H.J., S.M. Gross, D.M. Danks, and A.G. Wedd. 1990. Role of albumin's specific copper binding site in copper uptake by mouse hepatocytes. Am. J. Physiol. 258G: 988-991.

McDowell, L.R. 1992. Minerals in Animal and Human Nutrition. Academic Press, Inc. San Diego, CA. pp.176-204.

Miller, E.R., H.D. Stowe, P.K. Ku and G.M. Hill. 1979. Copper and zinc in swine nutrition. In NFIA Literature Review on Copper and Zinc in Animal Nutrition. National Feed Ingredients Assoc., West Des Moines, IA.

Murata, F., Yoshida, K., Ohno, S., Nagata, T. 1978. Mucosubstances of the granulocytes studied by means of electron microscopic radioautography and X-ray microanalysis. Proc. 9th Int. Cong. Electron Microscopy 2, 454-455

Opsahl W., Zeronian H., Ellison M., Lewis D., Rucker R. B., Riggins R. S. 1982. Role of copper in collagen crosslinking and its influence on selected mechanical properties of chick bone and tendon. J. Nutr. 1982;112:708-716

Owen, C.A.J. 1980. Copper and hepatic function. Ciba Found Symp. 79: 267-282.

Pond, W.G., D.C. Church, K.R. Pond, P.A. Schoknecht. 2005 Basic A nimal Nutrition and Feeding. pp. 185.

Pope, A.L. 1971. Review of recent mineral research with sheep. J. Anim. Sci. 33: 1332-1343.

SAS. 2010. SAS/STAT User's Guide (Release 9.2). SAS Inst. Inc, Cary, NC.

Schmitt, R.C., H.M. Darwish, J.C. Cheney, and M.J. Ettinger. 1983. Copper transport kinetics by isolated rat hepatocytes. Am. J. Physiol. 244: G183-191.

Smith, S.V. 2004. Molecular imaging with copper-64, Journal of Inorganic Biochemistry. Vol. 98: 1874-1901.

Spears, J.W. 1996. Organic trace minerals in ruminant nutrition. Animal Feeding Science and Technology. 58: 151-163.

Stansbury, W.F., L.F. Tribble, and D.E. Orr, Jr. 1990. Effect of chelated copper sources on performance of nursery and growing pigs. J. Anim. Sci. 68:1318-1322.

Strickland, G.T., W.M. Beckner, M.L. Leu, S. O'Reily. 1972. Turnover studies of copper in homozygotes and heterozygotes for Wilson's disease and controls: Isotope tracer studies with Cu. Clin. Sci. 43: 605.

Tangerman, A., Shaik, A.V., and Hoek, E.W.V.D. 1986. Analysis of conjugated and unconjugated bile acids in serum and jejunal fluid of normal subjects. Clinica Chemica Acta, 159: 123-132

Teague, H.S., L.E. Carpenter. 1951. The demonstration of copper deficiency in young growing pigs. J. Nutr. 43, p. 389.

Underwood, E.J. 1977. Trace Elements in human and animal nutrition, 4th ed. Academic Press, New York, NY. pp. 545.

Underwood, E.J. 1981. The mineral nutrition of livestock. Commonwealth Agricultural Bureaux, England. Pp. 91-113.

Underwood, E.J., and N.F. Suttle. 1999. The mineral nutrition of livestock. 3rd Edition. CABI Publishing, New York.

Wallace, H.D. 1968. High level copper in swine feeding., Int. Copper Res. Ass. Inc., N.Y.

Winge, D.R. and R.K. Mehra. 1990. Host defenses against copper toxicity. Int. Rev. Exp. Pathol. 31: 47-83.

Zhou, W., E.T. Kornegay, M.D. Lindemann, J.W. Swinkels, M.K. Welten, E.A. Wong. 1994. Stimulation of growth by intravenous injection of copper in weanling pigs. J Anim Sci. 72:2395-2403

Zimmerman, A.W., K.M. Hambidge, M.L. Lepow, R.D. Greenber, M.L. Stover, C.E. Casey. 1976. Pediatrics 69. Pp.176.

Table 1. Composition of basal diet, % DM

Corn	55.60
SBM	25.00
Dairy-Lac80	6.10
Porcine Meat & Bone	4.64
Fishmeal	3.20
Fat, Vegetable	3.00
MonoCal	0.38
Limestone	0.02
CTC (50 g/lbs)	0.40
Denaguard (10 g/lbs)	0.17
Lys	0.30
Thr	0.10
DL-Met	0.18
MHA	0.00
Salt	0.40
DynaK	0.10
Vitamin	0.25
Mineral-Cu Free	0.15
Nutrient profile	
ME, kcal/kg	3455.0
CP, %	21.79
Ca, %	0.8
P, % AV	0.45
Lys, Tot	1.44
Lys, TID	1.3
TID TSAA/Lys	60
Thr/Lys	65
Trp/Lys	15

Table 2. Effects copper concentration and source on performance of nursery swine.

	Treatment						Contrasts P <		
Item	Control	250 $CuSO_4$	75 Mintrex	75 $CuSO_4$	SEM	P <	Org vs. inorg	Cont vs. 250	Cu vs. No Cu
BW, kg									
Initial	6.11	6.20	6.13	6.04	0.56	0.99	0.91	0.91	0.99
D10	8.40	9.01	8.37	8.54	0.80	0.94	0.88	0.59	0.80
Final BW, kg	12.51	13.93	13.22	12.86	1.13	0.98	0.95	0.73	0.86
ADG									
D 0-10, kg/d	0.23	0.28	0.22	0.25	0.03	0.10	0.48	0.06	0.44
D11-22, kg/d	0.34	0.41	0.39	0.40	0.04	0.21	0.61	0.16	0.21
Overall, kg/d	0.29	0.35	0.32	0.31	0.03	0.91	0.79	0.56	0.58
Gain/feed									
D 0-10	0.68	0.84	0.70	0.71	0.01	0.02	0.56	0.01	0.04
D 11-22	0.64	0.71	0.68	0.66	0.02	0.04	0.74	0.04	0.03
Overall	0.66	0.78	0.69	0.69	0.02	0.06	0.59	0.05	0.09

Table 3. Effects copper concentration and source on plasma copper concentrations and bile components and antimicrobial effects of bile in nursery swine.

	Treatment						Contrasts P <		
Item	Control	250 $CuSO_4$	75 Mintrex	75 $CuSO_4$	SEM	P <	Org vs. inorg	Cont vs. 250	Cu vs. No Cu
Plasma Cu, mg/L	1.16	1.21	1.15	1.09	0.03	0.10	0.17	0.24	0.92
Bile									
Cu, mg/L	2.06	7.05	2.67	2.17	0.49	0.01	0.28	0.001	0.003
Conjugated bile acid, mg/dl***	240.5	250.8	270.9	250.2	31.0	0.54	0.87	0.94	0.97
pH	6.91	6.89	6.90	6.92	0.12	0.98	0.94	0.97	0.95
Total Cholesterol, mmol/l	4.21	4.15	4.18	4.05	0.56	0.78	0.84	0.87	0.93
Cholic acid, mmol/l	0.54	0.61	0.53	0.62	0.09	0.82	0.76	0.68	0.74
Chenodeoxycholic acid, mmol/l	37.6	39.5	38.1	37.2	2.1	0.75	0.98	0.48	0.70
Freq. of inhibition, +inhib./n	0/6	4/6	3/6	0/6	---	---	---	---	---

Table 4. Effects copper concentration and source on microbial populations in the small and large intestine. Log transformed.

Item	Treatment						Contrasts *P* <		
	Control	250 $CuSO_4$	75 Mintrex	75 $CuSO_4$	SEM	P <	Org vs. inorg	Cont vs. 250	Cu vs. No Cu
E.coli									
small	6.1	5.6	5.6	5.7	0.45	0.80	0.99	0.48	0.34
large	5.9	6.4	5.5	5.7	0.39	0.41	0.83	0.34	0.97
Colif.									
small	---	---	---	---	---	---	---	---	---
large	4.4	5.1	5.2	4.9	0.44	0.73	0.68	0.33	0.32
Enterobacter.									
small	6.7	6.2	5.5	6.1	0.36	0.23	0.26	0.05	0.10
large	6.5	6.8	5.7	6.1	0.37	0.24	0.46	0.16	0.53
Lactob.									
small	7.3	7.6	7.7	7.3	0.25	0.56	0.27	0.27	0.42
large	7.8	8.9	8.4	7.7	0.46	0.51	0.17	0.20	0.11