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The effect of dietary copper deficiency on plasma lipoprotein profiles in male and female hamsters

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The University of Arizona, 1991

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**THE EFFECT OF DIETARY COPPER DEFICIENCY
ON PLASMA LIPOPROTEIN PROFILES
IN MALE AND FEMALE HAMSTERS**

by

Denise Marie Surina

**A Thesis Submitted to the Faculty of the
COMMITTEE ON NUTRITIONAL SCIENCES
In Partial Fulfillment of the Requirements
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In the Graduate College
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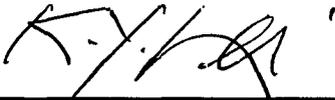
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ABSTRACT

Two studies were conducted to determine the conditions in which alterations in cholesterol and lipoprotein metabolism can be induced by copper deficiency in Golden-Syrian hamsters. Male hamsters were used in Study 1, and female hamsters were used in Study 2. Three week old animals were randomly assigned to one of two dietary copper treatments, deficient or adequate. In Study 1, body weight gain and food intake were suppressed in the animals fed the copper-deficient diet. In both studies, the hematocrit and hepatic copper content were significantly reduced in the treatment animals, and the plasma volume was enlarged. These changes, however, were small in comparison to the findings of previous studies using copper-deficient rats. The hamsters, therefore, appeared to be only marginally deficient. Despite the indications of marginal copper-deficiency, there were no differences in the composition of the various lipoprotein fractions between the two treatments, except for decreases in HDL protein concentration and pool size in the plasma of females fed the copper-deficient diet. It was concluded that in both studies the copper status of the treatment animals was not sufficiently diminished to affect lipoprotein profiles and plasma cholesterol. It was suggested that initiation of the dietary treatment should begin earlier than the third week of age.

INTRODUCTION

Copper is an essential mineral in animal diets that is necessary for the disulfide bonding of keratin, and serves as a vital component of copper metalloenzymes. Inadequate copper intake or absorption, excessive excretion, impaired utilization, or increased requirement can result in copper deficiency. Among other ensuing metabolic alterations, hypercholesterolemia has been described in many species, including humans, to result from copper deficiency. The increase in plasma cholesterol tends to coincide with the severity of the deficiency, and is accompanied by modifications in the composition and metabolism of the lipoprotein particles (Lei, 1990a). These particles transport the cholesterol and lipids in the plasma.

Investigations on the etiology of the deficiency-induced rise in plasma cholesterol and altered lipoprotein profiles have predominantly used rats as the experimental animal. These studies suggested that copper deficiency does not affect cholesterol degradation and excretion (Lei, 1978). On the other hand, cholesterol and fatty acid synthesis have been identified to increase in deficiency (Lei, 1978). An increase was also seen in the activity of HMG-CoA reductase (Yount et al., 1990), which is the catalyst in the rate-limiting and resolute step in cholesterol synthesis. Furthermore, it was found that newly synthesized cholesteryl esters are cleared from the liver faster in copper-deficient than in adequate rats (Shao & Lei, 1990), and that the amount transferred to the plasma is magnified two-fold (Yount et al., 1989). In concert, the hepatic cholesterol concentration is reduced (Lei, 1990b).

The quantity of cholesterol in low density lipoprotein (LDL) particles, has been described to increase about two-fold in the deficient animal (Lei et al., 1983). However, in rats, the rise in plasma cholesterol appears to be primarily localized in the high density lipoprotein (HDL) fraction (Lei & Carr, 1990). The protein content of HDL is also inflated, and most of the increase in both HDL protein and cholesterol is in the apo-E rich subfraction (Crowell & Lei, 1989). Copper deficiency also seems to enhance the removal of HDL cholesteryl ester from the plasma, especially to the liver (Carr & Lei, 1989), and to enhance the binding of apo E-rich HDL to specific uptake sites (Hassel et al., 1988).

Based on these findings, a theory was proposed (Lei, 1977, 1990b; Lei & Carr, 1990) that liver cholesterol is shifted to the serum pool, rendering low hepatic cholesterol levels, which are able to up-regulate hepatic HMG-CoA reductase activity (Brown & Goldstein, 1986). The stimulated reductase activity prompts cholesterol synthesis, followed by enhanced lipoprotein production and secretion, which are supported by increased fatty acid synthesis and increased amounts of cholesterol delivered to the liver by HDL. A prolonged net efflux of cholesterol from the liver to the plasma by these means may evoke the hypercholesterolemia observed in copper-deficient animals.

The few controlled studies on humans, which delineated the alterations in cholesterol and lipoprotein metabolism as a result of copper deficiency, indicate some differences from the rat. Humans carry the majority of cholesterol in LDL (Salter & Brindley, 1988), and in deficiency, the increase in plasma cholesterol is primarily localized in the LDL fraction (Lei & Carr,

1990). In contrast, rats carry the majority of cholesterol in the HDL fraction regardless of copper deficiency, and the LDL concentration in the serum is relatively small (Chapman, 1980). LDL is generated from very low density lipoproteins (VLDL). After the triglycerides in the core of the VLDL are hydrolyzed by lipoprotein lipase (LPL) and surface components are lost, the particle transforms into an intermediate density lipoprotein (IDL). IDL particles can be removed by the liver or remain in the circulation until they are further converted to LDL. The quantity of particles formed by this VLDL-IDL-LDL cascade varies considerably among species, which is one underlying reason for the differences between humans and rats in cholesterol dispersion among lipoprotein classes (Hay et al., 1986). The larger the VLDL particle, the more likely it is cleared from the plasma before being converted to LDL (Hay et al., 1986). Animals such as the rat have larger VLDL particles than humans, and thus lower LDL levels and less LDL-carried cholesterol.

Rats are additionally exceptional in that they can produce VLDL that include apo B₄₈, which is removed by the liver at a faster rate than the usual VLDL with B₁₀₀. In rats 90% of the VLDL is cleared from the plasma before transformation into LDL, whereas in humans only 50% of the VLDL is cleared (Hay et al., 1986). Another difference between the metabolism in the rat and in humans is that the rat can adapt its rates of cholesterol synthesis and degradation to a large extent, whereas humans have a much lower capacity (Spady et al., 1986). The male hamster is an animal that is very similar to man in this respect (Spady et al., 1986). In addition, hamsters are more similar than rats are to humans in the rate of LDL clearance and

cholesterol synthesis by the adrenal glands (Spady & Dietschy, 1985) and by the liver, which are the paramount organs of LDL uptake and cholesterol usage. Although hamsters are "HDL mammals" like rats, it is thought that they carry more cholesterol in the LDL fraction and have greater amounts of LDL than rats do. Accordingly, this rodent may fare to be a better model for inferential studies on lipoprotein and cholesterol metabolism, and hence, the related effects of copper deficiency. The purpose of the following two studies are to determine the parameters in which alterations in cholesterol and lipoprotein metabolism can be induced in male and female hamsters by copper deficiency.

LITERATURE REVIEW

The Function and Metabolism of Plasma Lipoproteins

Animal blood is composed of hematocrit and plasma. The hematocrit consists of red and white blood cells, and platelets, whereas the plasma is mainly water with proteins, hormones, antibodies, nitrogenous waste, electrolytes, nutrients and clotting factors (Purves & Orians, 1983). Without the clotting factors, the plasma is called blood serum. One of the main functions of the blood serum is to transport nutrients, making them available to the other body cells (Vander et al., 1970). Yet, triglycerides and cholesterol are not soluble in the water base medium of the blood, and therefore, require a packaging system for their transportation. The packaging units are lipoprotein particles, that not only allow for a large quantity of lipids to be transported through the blood, but also to be targeted to specific cells.

Classification and Separation

The plasma lipoproteins have commonly been classified into five major groups: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (Havel, 1987; Nestel, 1987; Scanu, 1986). The different lipoprotein groups vary in composition of fats and proteins, and in size and density (Table 1); yet, all are structurally similar. They have a hydrophobic core composed of triglycerides and cholesteryl esters that is enclosed by a spherical, relatively hydrophilic surface layer of proteins, phospholipids and unesterified cholesterol (Havel, 1987). The hydrophobic regions of the surface

Table 1. Properties of Human Plasma Lipoprotein Classes¹

Class	Sources	Density Range (g/ml)	Diameter (nm)	MW (dalton x 10 ⁶)
Chyl	Intestine	0.94	80-500	400
VLDL	Liver, intestine	0.940-1.006	30-80	18-80
IDL	VLDL, chyl	1.006-1.019	25-30	5-10
LDL	IDL	1.019-1.063	16-25	2.5
HDL	Liver, intestine	1.063-1.210	7-13	0.2-0.4

¹Table adapted from Salter and Brindley (1988) and Gotto et al. (1986).

components are aligned toward the core. The surface proteins are termed apoproteins and provide stability to the particle (Vander et al., 1970). In addition, they are involved in the targeted delivery of the lipoprotein particles and their contents to specific cells and tissues by enzyme activation. Table 2 outlines the established apoproteins and their known functions.

The classification of plasma lipoproteins is based on their flotation density in the ultracentrifuge. The characteristic densities and flotation rates are related to, particularly, their neutral lipid content and the ratio of protein to lipid. The classes exhibit density heterogeneity and differences in size and protein, lipid distribution; yet there are still broad characteristics within a single lipoprotein class (Scanu, 1986). Changes in size and composition occur progressively during metabolism by the transfer and exchange of both surface and core components from one particle and class to another (Havel, 1987). Serum lipoprotein fractions can be separated by isopycnic density gradient ultracentrifugation, or by their electrophoretic migration, or based on their size by gel-filtration (Scanu, 1986). The characteristics of the five lipoprotein classes are summarized in Table 1.

Chylomicrons

The largest, most lipid containing, and least dense lipoprotein particles are the chylomicrons, which are synthesized in the intestines in response to dietary fat (Miller & Small, 1987). The main function of the chylomicrons are to transport and deliver dietary triglycerides to extrahepatic tissue for energy

Table 2. Physical Properties of the Major Apoproteins of Human Serum Lipoproteins¹

Apo	MW (daltons)	Sources	Lipoprotein Distribution	Function
A-I	28.000	Intestine, liver	HDL ₃ , Chyl	LCAT cofactor
A-II	17.000	Intestine, liver	HDL ₃ , Chyl	Hepatic Lipase activator (?)
A-IV	46.000	Intestine, liver	HDL, Chyl	LCAT cofactor (?)
B-48 and	200.000	Intestine	Chyl	Chylomicron synthesis secretion
B-100	400.000	Liver	VLDL, HDL	VLDL synthesis, secretion; receptor binding
C-I	5.800	Liver	Chyl, VLDL, HDL	LCAT cofactor (?)
C-II	9.100	Liver	Chyl, VLDL, HDL	Lipoprotein lipase (LPL) cofactor
C-III	8.750	Liver	Chyl, VLDL, HDL	Inhibitor of LPL and of premature remnant clearance
D	22.000	Liver	HDL	Chol ester exchange (?)
E	35.000	Liver	Chyl, VLDL, HDL	Receptor binding

¹Table adapted from Havel (1987) and Dolphin (1985).

and storage, and to deliver dietary and endogenous cholesterol to the liver. Dietary triglycerides, after being hydrolyzed to fatty acids and monoglycerides, diffuse across the intestinal wall and are resynthesized to triglycerides within the mucosal cells (Kapit et al., 1987; Linscheer & Vergroesen, 1988). Dietary and endogenous cholesterol, as well as fat-soluble vitamins, also pass across the intestinal wall into the mucosal cells, where they are packaged along with the triglycerides into chylomicrons (Kapit et al., 1987). The apoproteins associated with this lipoprotein are apo B₄₈ and the A apoproteins, all of which are also synthesized in the intestine (Miller & Small, 1987). The size and relative composition of the chylomicron particles are dependent on the amount of fat consumed and the synthesis rates of the apoproteins (Salter & Brindley, 1988).

Once synthesized, chylomicrons are released into the lymphatic circulation and are transported through the thoracic duct to the bloodstream. There they acquire apo E and apo C_I, II, and III from HDL and donate apo A_{IV} to HDL particles (Hay et al., 1986; Miller & Small, 1987). When the chylomicrons pass through the bloodstream, their newly acquired apo C_{II} is able to bind and activate the extrahepatic enzyme lipoprotein lipase (LPL). This enzyme hydrolyzes the triglycerides of the particle to glycerol and fatty acids, both of which are small enough to pass into the proximal cells of the tissue to which LPL is attached (Garfinkel & Schotz, 1987). Although LPL appears to be ubiquitous, the predominant sites of hydrolysis and uptake are the adipose tissue where triglycerides are stored, and the heart and skeletal muscle where triglycerides are oxidized for energy (Borensztajn, 1987; Eckel, 1987; Garfinkel

& Schotz, 1987). As the lipids are transferred to the extrahepatic tissues, the particle becomes smaller. In conjunction, the surface layer decreases by transferring the A and C apoproteins and most of the phospholipids to HDL. When the particle loses apo C_{II}, its affinity for LPL decreases, and it recirculates in the blood as a chylomicron remnant containing mainly cholesterol esters (Windler et al., 1980, as cited in Windler et al., 1988). Without the C apoproteins, the remnant rapidly binds to the hepatic apo E receptor and is internalized and hydrolyzed by receptor mediated endocytosis (Goldstein and Brown, 1986).

Very Low Density Lipoproteins

The second largest lipid containing lipoprotein is VLDL. These particles are formed in the liver and consist of triglycerides, cholesteryl esters, phospholipids, apo E, a single apo B₁₀₀, and some C apoproteins. The triglycerides come predominantly from hepatic fatty acid synthesis by the liver, and the cholesterol is derived from hepatic de novo synthesis or from extrahepatic sources (Hay et al., 1986). When the VLDL particles are released from the liver, they circulate in the blood where their triglycerides are hydrolyzed by endothelial LPL, for which apo C_{II} is a cofactor, and by hepatic lipase, which is independent of apo C_{II}. The lipolysis by LPL is common to both VLDL and chylomicrons; yet the VLDL particles are metabolized more slowly because they are usually smaller, and thus, do not bind as easily to the enzyme (Garfinkel & Schotz, 1987; Nestel, 1987). Upon hydrolysis, VLDL releases apo C to HDL in exchange for cholesterol esters and triglycerides, and whereby transforms into a series of IDL particles (Nestel, 1987).

Intermediate Density Lipoproteins

The IDL particles are smaller in size and more dense than the VLDL particles, and contain apo B₁₀₀, apo E, and some C apoproteins. These particles can be taken up by the apo B, E receptors of the liver via receptor mediated endocytosis. The particles that are not cleared from the blood in this manner remain in the circulation and eventually transform into LDL particles by losing more triglycerides, and transferring phospholipids, free cholesterol, apo E, and apo C to HDL particles (Hay et al., 1986). By the action of the enzyme lecithin-cholesterol ester transferase (LCAT), the free cholesterol of HDL is esterified and returned to the evolved LDL particle (Hay et al., 1986; Nestel, 1987).

Low Density Lipoproteins

LDL particles contain a single B₁₀₀ apoprotein and have the largest proportion of cholesterol esters of any other lipoprotein type. These particles function to transport and deliver endogenous cholesterol. Whether these lipoproteins can be produced by means other than the VLDL-IDL-LDL cascade is still uncertain. Most tissues are able to take in LDL from the blood; yet the majority of LDL is removed by only a few organs (Hay et al., 1986). A specific regulated, high-affinity receptor for LDL exists (the apo B, E receptor) and is responsible for two-thirds of LDL catabolism (Nestel, 1987). Receptor-independent uptake of LDL is unregulated and is a function of the circulating LDL concentration. The liver is the primary site of receptor- and non-receptor-mediated removal, accounting for about half of the total LDL degradation (Nestel, 1987). The liver requires cholesterol for bile acid and

lipoprotein synthesis, besides the usual need for membrane formation and repair. The adrenal and gonads use the receptor pathway of uptake and are the most active sites, deriving cholesterol for steroid hormone production. The intestine also clears a recognizable amount of LDL from the blood, about a tenth of the total LDL removed. The adipose tissue, skin, and muscle, which all require cholesterol for cell genesis and maintenance, use the non-receptor- and receptor-mediated pathways to an equal degree (Nestel, 1987).

There is a third pathway for LDL removal called the scavenger pathway, which occurs in endothelial cells and macrophages . This pathway is used for modified LDL particles and provides a means of uptake into arterial tissues (Gianturco & Bradley, 1987). It is thought that this pathway is involved in the genesis of coronary heart disease, which is the cause of half of all the deaths in the U.S. (Brown and Goldstein, 1985). Furthermore, elevated levels of plasma LDL have been linked to an increased risk of atherosclerosis. This disease is attributed to an accumulation of cholesterol in the wall of arteries due to endothelial cell injury. The injury is thought to alter the permeability of lipoproteins (Shasby, 1982). LDL is a key lipoprotein in this disease because of its size and, thus, permeability, and its cholesterol content. When the LDL particles that enter the injured area are oxidatively modified, they are susceptible to the scavenger pathway of uptake by resident macrophages. This uptake system is not regulated, and the macrophages can therefore become overloaded with cholesterol and form foam cells, which in turn lead to plaques (Gianturco & Bradley, 1987). These plaques can inhibit blood flow

and cause clotting, and can obstruct an artery and cause a heart attack or a stroke (Brown and Goldstein, 1985).

High Density Lipoproteins

HDL are the most dense of the lipoproteins, and contain the least proportion of lipids and the largest proportion of phospholipids compared to the other lipoproteins. It is speculated that nascent discoidal HDL contain only apo E, and originate from both the liver and intestine, and from the complexing of components in the circulation (Patsch & Gotto, 1987). The accumulation of apo A_I and A_{II} from chylomicrons, and the exchange of apo E for apo C with VLDL converts the particle into a mature form (Hay et al., 1986). There are two major subclasses of mature HDL, HDL₂ and HDL₃, which differ in size and apoprotein content. It is thought that the smaller, more dense HDL₃ is transformed into HDL₂ upon acceptance of phospholipids, cholesterol, and core lipids from triglyceride-rich lipoproteins that undergo lipolysis by LPL. HDL₂ can be recycled back to HDL₃ by the lipolytic action of hepatic lipase, or by the transfer of core lipids to VLDL or LDL (Nestle, 1987; Patsch & Gotto, 1987). Another subclass of HDL exists under certain conditions (e.g. cholesterol feeding). These particles have a low density, are similar to LDL in size and cholesterol composition, and are enriched in apo E (Hay et al., 1986; Patsch & Gotto, 1987). They are referred to as apo E-rich HDL, HDL₁, or HDL_C.

HDL does not possess any immobile components and has a key role in the regulation of lipoprotein catabolism as a receptor and donor of lipids and proteins (Hay et al., 1986). It is found in association with the enzyme LCAT

because it is the source of one of the enzyme's cofactors, apo AI (Jonas, 1987). LCAT is responsible for the synthesis of nearly all plasma cholesteryl esters by catalyzing the transfer of fatty acids from lecithin to the hydroxyl group on cholesterol (Havel, 1987). HDL provides the cholesterol and phospholipids, which are obtained from the other lipoprotein classes, for the esterification by LCAT. HDL is also associated with lipid transfer proteins and, hence, the redistribution of the cholesteryl esters among the other lipoproteins. In addition, HDL has the capability of extracting cholesterol from the plasma membranes of extrahepatic tissues (Havel, 1987; Hay et al., 1986; Patsch & Gotto, 1987). Therefore, cellular free cholesterol which is taken up by HDL, esterified by LCAT, and transferred to LDL or VLDL, has the potential to be delivered to the liver and to other tissues that remove LDL and VLDL. This overall process is termed "reverse cholesterol transport" to emphasize the delivery of cholesterol from peripheral tissues to the liver. Yet, this process also has the capability to transfer cholesterol from one extrahepatic tissue to another.

The liver, intestine, kidney, adipocytes, fibroblasts, and adrenal cortical cells appear to bind, internalize and degrade apo E free HDL (Nestel, 1987). As with LDL, the liver is the major uptake site of HDL and HDL cholesteryl esters, and the adrenals and gonads have the greatest uptake per weight. The kidney is the second largest site of degradation, and other tissue (i.e. gut, muscle, skin) remove only minor amounts of HDL (Nestel, 1987). Preferential removal of protein and cholesteryl esters may occur and is possibly regulated by the cholesterol status of the cell (Carr & Lei, 1990).

Copper

Copper is a metallic element that occurs naturally in two isotopes: ^{63}Cu (69.1%) and ^{65}Cu (30.9%), and exists in two oxidation states: cuprous (CuI) and cupric (CuII). There are also two radioisotopes of the metal, ^{64}Cu and ^{67}Cu , which have been employed in biological studies as tracers. In the 1920s and 1930s, copper became recognized as an essential nutrient in animal diets from studies on laboratory rats and farm animals. The essentiality was officially established when Hart et al. (1928) demonstrated the necessity of copper for the prevention of anemia in the rat. The necessity was attributed to the mineral's role in hemoglobin formation. Since then, balance studies in healthy human adults have determined that 1.5 to 2.0 mg of copper are lost per day primarily via feces (Danks, 1988). In order to replenish the loss, a daily intake of 30 $\mu\text{g}/\text{kg}$ body weight for males (Solomons, 1980) and 80 $\mu\text{g}/\text{kg}$ body weight for infants (Davis & Mertz, 1987), which includes a margin of safety, has been recommended by the WHO Expert Committee. During the rapid growth period after the sixth month, copper is needed in an infant's diet to sustain growth.

The healthy adult male human has been estimated to contain 70 to 80 mg of total-body copper (Solomons, 1988). Newborns and very young animals normally have a larger copper to body weight ratio than the adults of the same species. This higher copper level is maintained throughout the suckling period and then decreases steadily during growth until an adult level is obtained (Davis & Mertz, 1987). The distribution of total-body copper among tissues varies with species, age and copper status (Davis & Mertz, 1987;

Underwood, 1977). Yet, in general, the concentration of copper in the liver, brain, kidneys, heart, blood and hair are high. The pancreas, skin, muscle, spleen, and bones contain a relatively intermediate amount of copper. And, examples of organs that have much lower concentrations of copper are the pituitary, thyroid, thymus, prostate gland, the ovary and the testis (Carlton and Henderson, 1963, as cited in Underwood, 1977).

Copper Metabolism

Food sources that contain a high level of copper include oysters, shellfish, liver, dried fruits, nuts and legumes. Yet, in an average human diet, potatoes, fruit, bread, and meats are the principle contributors of copper (Danks, 1988; Delves, 1980; Solomons, 1980). The copper present in foods is in bound form and requires digestion of the food for its dissociation and absorption (Solomons, 1988). After binding to salivary and gastric secretions, copper complexes tend to dissociate in the acidic environment of the lumen (Solomons, 1988).

Absorption

Absorption occurs in the stomach and various parts of the small intestine, with maximal absorption in the upper duodenum (Danks, 1988; Hsieh & Hsu, 1980; Underwood, 1977). Other segments of the gastrointestinal tract are also capable of absorbing copper depending on the species and age (Davis & Mertz, 1987). Thirty to fifty percent of dietary copper is absorbed (Danks, 1988; Hsieh & Hsu, 1980), though this is influenced by a number of factors and can vary between 25 to 75% (Davis & Mertz, 1987).

Although controversial, the chemical form of copper has been shown to affect absorption. By studying the appearance of ^{64}Cu in cattle blood, Chapman and Bell (1963, as cited in Underwood, 1977) reported that specific chemical forms of the mineral are absorbed at the following relative rate: $\text{CuCO}_3 > \text{Cu}(\text{NO}_3)_2 > \text{CuSO}_4 > \text{CuCl}_2 > \text{Cu}_2\text{O} > \text{CuO} > \text{Cu}$. Other studies, which have been outlined by Davis and Mertz (1987) and by Underwood (1977), have used differences in biological copper to support the claim that chemical form influences the availability of copper for absorption.

Copper absorption is also regulated by the nutritional status and possibly the age of the individual (Davis & Mertz, 1987). For example, copper deficiency has the effect in several species of increasing absorption. And young sucklings have been shown to have a higher absorption rate than mature animals of the same species. Pregnancy may also affect the absorption of copper favorably. Pregnant women retain more copper than nonpregnant women, which is thought to be due to greater absorption and diminished excretion (King & Wright, 1985, as cited in Davis & Mertz, 1987). The rise in copper uptake may result from an increased demand for copper, and because estrogen inhibits uptake, the rise is not due to hormonal changes that accompany the pregnant state (Bremner, 1980).

Dietary factors seem to play an equally important role in copper uptake. Some amino acids stimulate copper absorption (Davis & Mertz, 1987). Other amino acids (Underwood, 1977), possibly fructose (Davis & Mertz, 1987), and dietary fiber (Danks, 1988; Solomons, 1980), as well as high dietary levels of ascorbic acid (Davis & Mertz, 1987) decrease the availability of copper.

Ascorbic acid may do this by reducing the copper ion or by forming a stable complex with it. In addition to the dietary influences, endogenous compounds that depress copper absorption are present in bile, pancreatic juice, and other intestinal and gastric secretions (Davis & Mertz, 1987).

Transition metals zinc, cadmium, calcium, lead, and possibly mercury compete for uptake binding sites in the intestine and, consequently, interfere with copper absorption (Hsieh & Hsu, 1980). These metals also affect the utilization of copper by minimizing its solubility within the intestinal lumen (Mills, 1980). The antagonistic effect that zinc has on copper has been well established (Davis & Mertz, 1987). This effect has received the most attention compared to that of the other interfering metals because zinc and copper share a similar intermediate transport and storage mechanism. (This mechanism will be described in the next subsection.)

There are two means by which copper can be taken up by the intestine: passive diffusion and active transport. The energy-dependent transport is the minor route and requires a copper-amino acid complex (Hsieh & Hsu, 1980; Solomon, 1988). Specifically, copper complexes to histidine and is absorbed by the histidine amino acid carrier (Solomons, 1988). This mechanism is important when copper concentrations are low (Danks, 1988). The major route of uptake is diffusion and because it is saturable, it is most likely carrier-mediated. Further studies clarified that it is not glucose-dependent-mediated (Solomon, 1988). This mechanism occurs at high concentrations of copper (Danks, 1988) and is less affected by metabolic inhibitors (Bremner, 1980).

Moreover, it may be responsible for the uptake of ionic copper (Bremner, 1980).

Intermediary Metabolism

The primary control point in copper metabolism is the transport of copper from the mucosal to the serosal side of the intestine (Solomons, 1988). The protein metallothionein has been implicated in this regulation (Bremner, 1980; Danks, 1988; Davis & Mertz, 1987; Solomon, 1988). Metallothionein may also act to protect the cell from possible damaging effects of metals (Evans & Johnson, 1978, as cited in Bremner, 1980) and to regulate absorption in response to changes in dietary intake of copper (Davis & Mertz, 1987).

Furthermore, it has been suggested that metallothionein is the mediator of copper and zinc interactions (Cousins, 1985, as cited in Davis & Mertz, 1987). Synthesis of the protein is induced by copper and zinc (Bremner, 1980), though zinc is a stronger inducer (Danks, 1980). Metallothionein complexes do not exit the mucosal cell into the blood, and therefore, metals bound to the protein are stopped from entering into the portal circulation (Bremner, 1980). High levels of this protein are especially conducive to restraining copper and zinc in the intestines. However, copper has greater affinity than zinc for the binding site of metallothionein and accordingly, is able to displace zinc (Bremner, 1980). This leads to a form of copper that is not readily transported into the blood, regardless of whether the metal is in excess or not.

Consequently, large doses of zinc induce synthesis of metallothionein, that preferentially bind and trap copper in the intestinal mucosal cells until the cell is sloughed off and the metals become part of the intestinal contents

again. The number of metallothioneins vary in species. Humans have five (Danks, 1988).

After transport to the serosal side of the intestinal mucosal, absorbed copper seems to become loosely associated with albumin and, to a lesser extent, certain amino acids (i.e. histidine, threonine, and glutamine) in the plasma (Bremner, 1980; Hsieh & Hsu, 1980). These forms are distributed to the tissues and can readily pass into the erythrocytes (Underwood, 1977). Almost all of the copper is deposited in the liver, which is the main storage organ of the body and the central organ of copper metabolism (Hsieh & Hsu, 1980). Yet, hepatocytes do not obtain copper directly from albumin, but may use histidine as a delivery service (Danks, 1988). Danks (1988) went so far as to speculate that albumin may merely act as a transient store of copper in the bloodstream and that cell uptake of copper is via histidine complexes. This idea is in line with the findings that uptake is rapid, saturable, temperature dependent, not inhibited by certain poisons, and not affected by other typically competitive metal ions (Davis & Mertz, 1987). These findings point to the conclusion that uptake is via specific, facilitated diffusion through the formation of a specific amino acid complex.

Once within the hepatocytes, copper is found in the mitochondria, microsomes, nuclei and soluble fractions of the parenchymal cells, but this varies with age, strain, and copper status of the animal (Underwood, 1977). Within these organelles, copper is incorporated into proteins such as superoxide dismutase, mitochondriocuprein, metallothionein, cytochrome c oxidase, or ceruloplasmin (Hsieh & Hsu, 1980). Bremner (1980) claims that

the first three may act as storage proteins. Superoxide dismutase accounts for 20 to 50% of total hepatic copper when copper concentration is low.

Metallothionein is hypothesized to function as a temporary storage site and a detoxification protein (Bremner, 1980). Similarly to intestinal metallothionein, the hepatic equivalent can be hyperinduced by excess zinc levels and can then trap copper, making it inaccessible to the other copper proteins in the liver (Danks, 1988). On the whole, metallothionein is one of the major components in copper metabolism.

Ceruloplasmin is another dominant component in copper metabolism and acts as the carrier and donor of copper exported from the liver to target tissues (Bremner, 1980). Eighty percent of the copper present in the plasma is contained in this protein (Danks, 1988). The enzyme is capable of oxidizing ferrous, which is important for the release of iron from stores, and is thus, the primary link between copper and iron metabolism and possibly between copper and hemoglobin formation (Frieden, 1980; Underwood, 1977).

Ceruloplasmin is also capable of oxidizing various amines, adrenalin and serotonin, and of acting as an endogenous modulator in the inflammatory response, and of scavenging superoxide radicals (Frieden, 1980). The synthesis of this protein is not entirely dependent on copper availability (Danks, 1988; Solomons, 1988). Production is stimulated by estrogen (Danks, 1988), and interestingly, females have higher plasma copper levels, as well as higher ceruloplasmin levels than males (Danks, 1988; Underwood, 1977).

With this information and the results of studies outlined in Underwood (1977), it is tempting to postulate that via ceruloplasmin, the higher estrogen

levels in females may be an explanation for the greater plasma copper concentration.

Excretion

The process of copper metabolism must be able to supply an adequate amount of copper to the body without an excess that would exert toxic effects. Thus, a balance between absorption and excretion must exist. The predominant means of copper excretion is via bile, and this constitutes the major homeostatic regulation of body copper (Solomons, 1988). Biliary copper is poorly absorbed by the intestine, and thus, there is little enterohepatic circulation. The copper in salivary, gastric, and intestinal juices that is not reabsorbed is excreted in the feces. Urine, sweat, hair, nails, skin, menstrual losses and lactated milk all contribute only minorly to copper loss (Davis & Mertz, 1987; Solomons, 1988; Underwood, 1977).

Functions of Copper

Copper is involved in the disulfide bonding of keratin; but, its predominant function is as a component of copper metalloenzymes (Danks, 1980, 1988; Solomons, 1988). All cuproenzymes mediate reactions that consume molecular oxygen or related compounds, though their substrates, reactions and metabolic roles are very different (Cass & Hill, 1980; Solomons, 1988). Copper is found in the active site of these enzymes, and additionally in other parts of some of the enzymes. Based on the attachment of copper to the active site, there are four types of cuproenzymes (Cass & Hill, 1980). The enzymes in group I and II each contain a single copper ion in a tetrahedral surrounding

and a square-planar arrangement, respectively. Those in group III have two ions situated close to one another; and those in group IV consist of multi-copper proteins. A summary of the enzymes, their function and the effect of copper deficiency can be found in Table 3. Of the listed enzymes, cytochrome c oxidase, ceruloplasmin, lysyl oxidase, and the monoamine oxidases are the four key contributors to the symptoms of deficiency (Davis & Mertz, 1987).

Copper Deficiency

Copper deficiency was recognized in humans in 1964 when seen in Peruvian children with Protein-energy malnutrition (PEM). These children had extreme diarrhea, were fed milk based diets, which do not provide copper, and obtained water from noncopper pipes. It was found that the dietary factors, malabsorption and malutilization were the culprits in the ensuing deficiency (Solomons, 1988). The discovery in 1972 of the genetic disorder Menke's disease, which impairs intestinal copper absorption and copper utilization, confirmed and established the effects of pure, severe copper deficiency in humans (Danks, 1988). It is now known that copper deficiency can occur due to decreased copper intake or absorption, increased excretion, impaired utilization, or increased requirement (Danks, 1980). Yet, in the medical literature, only a small number of patients have been reported deficient (Danks, 1988). As a note, the incidence of mild copper deficiency is difficult to determine and quite controversial (Davis & Mertz, 1987).

Dietary deficiency is usually only seen in premature babies, elderly patients, and in conditions of extremely high zinc intake (Danks, 1988). Premature

Table 3. The Functions of Copper Enzymes in Humans and the Effects of Copper Deficiency¹

Enzyme	Functional Role	Known and <i>Possible</i> consequence of deficiency
Cytochrome c oxidase	Electron-transport chain	<i>Muscle weakness; cardiomyopathy; brain degeneration</i>
Superoxide dismutase	Free radical detoxification	<i>Membrane damage; other free radical damage</i>
Tyrosinase	Melanin synthesis	Failure of pigmentation
Dopamine- β -hydroxylase	Catecholamine synthesis	<i>Neurological effects, type uncertain</i>
Lysyl oxidase	Cross-linking of collagen and elastin	Vascular rupture; loose skin and joints; osteoporosis; emphysema; bladder diverticulae
Ceruloplasmin	Ferroxidase, amine oxidase, Cu transport	<i>Anemia; deficient supply of copper to other tissues</i>
Clotting factor V	Blood clotting	<i>Bleeding tendency</i>
Unknown	Cross-linking of keratin (disulfide bonds)	Pili torti

¹Table from Danks (1988).

babies can become copper-deficient because of their rapid weight increase and low copper stores (Danks, 1980). Cow milk has a much lower accessible copper content than human milk, and babies (both premature and not) fed low copper formula cow milk are therefore susceptible to deficiency (Danks, 1980). Elderly patients with special medical ailments are also susceptible to deficiency (Danks, 1980). In former times, patients on prolonged parenteral nutrition became copper-deficient because of a lack of parenteral copper (Solomons, 1988). In addition to these situations, high levels of oral zinc intake cause deficiency by the mechanism described previously, which results in the entrapment of copper bound to metallothionein in the intestine and liver. Two other dietary/medicinal substances that can reduce copper absorption are antacids and ascorbic acid (Solomons, 1988). Whether severe copper deficiency can result from these last two mentioned substances is unclear.

Other than dietary means, a number of circumstances are able to induce copper deficiency. Prolonged diarrhea and aminoaciduria abate the uptake of copper in the intestines and, especially in the young, can lead to copper deficiency (Danks, 1980). In addition, disease states such as short-gut celiac sprue, jejunoileal bypass, gut resection, and phenylketonuria can interfere with copper absorption or utilization and provoke copper deficiency (Solomons, 1988). This knowledge emerged from human case studies. Of course, the effects of copper deficiency on organs have been documented mainly from laboratory animals. The liver copper concentration is the most sensitive and falls more rapidly than any other organ during deficiency

(Davis & Mertz, 1987). The blood copper concentration is also affected by deficiency, which is seen in eurythrocytes, ceruloplasmin, and direct reacting copper (Davis & Mertz, 1987).

Many of the symptoms of copper deficiency can be explained by the role copper plays in cuproenzymes and in the cross-linking of keratin (Table 3). At the same time, the prominent effects vary widely with species and the age at which deficiency is experienced (Danks, 1988).

When deficiency is prolonged, or the onset occurs early, anemia accompanied by neutropenia and bone changes occur in all animals (Danks, 1980; Underwood, 1977). Neutropenia is the earliest manifestation, later followed by anemia (Solomons, 1980). Copper plays several roles in iron metabolism that can be linked to anemia and neutropenia. Impaired ferroxidase activity of ceruloplasmin with consequent failure of iron mobilization from tissue stores is a partial explanation (Danks, 1980, 1988; Underwood, 1977). An extreme reduction of cytochrome c oxidase and superoxide dismutase was found in the bone marrow of copper-deficient mice, and a reduction of erythropoietin was found in deficient rats, both of which may help to explain the anemia and neutropenia (Danks, 1988). The bone changes observed are splaying of metaphyses, corner fractures, and skeletal demineralization, and are partly due to faulty cross-linking in elastin and collagen (Danks, 1980; Davis & Mertz, 1987). Scorbutic lesions occur and are very similar to those seen in scurvy. The similarity may be related to ceruloplasmin and its proposed involvement in ascorbic oxidase activity (Solomons, 1980; 1988). Regardless of age, osteoporosis has been reported in most copper-deficient

cases (Danks, 1988). As with the bone pathologies in general, this symptom is attributed to a lack of lysyl oxidase, resulting in defective elastin and poorly crossed collagen, which is broken down more rapidly than normal (Danks, 1988). Interestingly, an estrogen/copper interaction via ceruloplasmin may be linked to postmenopausal osteoporosis, but that is only speculation (Danks, 1988).

Changes in hair, fur, or wool, pigmentation and skin often accompany severe copper deficiency (Davis & Mertz, 1987). For instance, in congenital defects, a kinky, twisted hair syndrome, pili torti, occurs due to a defect in disulfide bridging of keratin (Underwood, 1977). The fine hair observed in deficient Peruvian children, however, is difficult to trace to copper because the symptom also occurs in grossly malnourished children (Danks, 1980). The loss of pigmentation is pronounced in most species and is based on a decrease in tyrosinase activity, and thereupon, a limit in melanin synthesis (Solomons, 1980). The changes in skin and joints originate from abnormal cross-linking of elastin and collagen, while the keratin changes may interfere with epidermal shedding and give rise to follicular hyperkeratosis (Danks, 1980, 1988).

In cases of inborn error and prenatal onset of deficiency, cerebral and neurological defects evolve (Danks, 1980; Solomons 1980). The neurological problems are predominantly localized in the cerebellum, motor cortex, and spinal tracts; yet, an overall loss in myelination does occur (Danks, 1988). These specifics are illustrated by the nervous disorder and conditional loss in muscle tone and movement. Neonatal ataxia, which is nicknamed swayback,

is an example of a nervous disorder that occurs in lambs, and is characterized by uncoordinated movement. It occurs in other farm animals as well when their pastures are low in attainable copper (Davis & Mertz, 1987). The relationship of copper deficiency to these disorders is ascribed to a decline in cytochrome c oxidase, and a depression of phospholipid synthesis (Underwood, 1977). The problems in the cerebellum and motor cortex may generate additionally from a decrease in dopa β -monooxygenase activity that leads to a deficiency in the neurotransmitters catecholamines, ultimately promoting brain lesions (Danks, 1980).

Copper deficiency causes cardiovascular disorders in some species. The first cases emerged in Australian cattle with a degeneration of the myocardium and fibrosis (Davis & Mertz, 1987). This degeneration was later reported to occur in pigs, rats, mice, guinea pigs, and chicks (Danks, 1980). A decrease in elastin content of the aorta, hypertrophy of the heart, diminished catecholamine levels, and abnormal collagen framework were also observed (Danks, 1988; Davis & Mertz, 1987). The characteristics of these disorders imply that deficiencies in cytochrome c oxidase, lysyl oxidase, and dopamine- β -hydroxylase are responsible (Danks, 1988; Davis & Mertz, 1987).

A decrease in cytochrome c oxidase is partially responsible for almost all of the ill effects of deficiency (Danks, 1980). The enzyme is involved in oxidative phosphorylation by the terminal electron transport chain. The symptoms hypothermia and hypotonia experienced with deficiency are thought to stem from the decreased oxidative phosphorylation (Solomons, 1988). Gallagher and Reeve (1971, as cited in Underwood, 1977) reported a

depression in phospholipid synthesis as a direct result of cytochrome c oxidase deficiency. The interpretation holds that mitochondria are simply unable to synthesize phospholipids without endogenous ATP (Davis & Mertz, 1987). There is also a decrease in glucose oxidation in the deficient state, which may explain the glucose intolerance that occurs. Yet for this symptom, the decrease in oxidative phosphorylation may not be the causal factor; insulin binding is possibly affected by copper and may precede an effect on glucose metabolism, but this is still uncertain (Davis & Mertz, 1987).

Alterations in the metabolism of lipids and cholesterol, and in peroxidation have been demonstrated in many studies using copper-deficient experimental animals (Davis & Mertz, 1987). The fatty acid composition of organs and organelles may change in the deficient state and contribute to elevated peroxidation (Danks, 1988). Hypercholesterolemia has been induced by deficiency in many species including monkeys, rats, rabbits and guinea pigs. The possible etiology of this phenomena, as well as a brief review of the effect copper deficiency has on lipoprotein profiles will be described in the next section.

Hypercholesterolemia and Copper Deficiency

In the earliest related studies, a high dietary ratio of zinc to copper was shown to induce hypercholesterolemia (Klevay, 1973). It was later clarified by large-scale factorial experiments, in which the levels of dietary copper and zinc were varied, that low copper intake was responsible for the observed hypercholesterolemia (Murthy & Petering, 1976; Petering et al., 1977). The

influence of zinc appeared to descend from the copper-zinc interrelationship. By now it is well founded that severe copper deficiency leads to altered cholesterol metabolism. The studies establishing that hypercholesterolemia results from deficiency have been reviewed by Lei (1990a). Although the majority of the studies have employed rats as the experimental subjects, an unequivocal, or at least a tendency of hypercholesterolemia due to copper deficiency has also been reported in mice, rabbits, monkeys, and humans. The effect does not appear to be gender-specific because it has been demonstrated in female rats and mice, as well as in the male aforementioned animals.

Most of the studies summarized by Lei (1990a) used weanling animals, except the experiments on humans and monkeys, and one on rats, which used older or adult animals. Despite the latter studies, it may be erroneous to assume that all animals would display hypercholesterolemia regardless of when deficiency commences. Especially after animals have accumulated large copper stores, the induction of a deficient state that is sufficient to enlarge the plasma cholesterol content may be difficult or require a longer treatment duration. This stands to reason considering that the increase in plasma cholesterol is thought to correspond to the severity of deficiency (Lei, 1990a).

The increase in plasma cholesterol concentration seen in copper deficiency is usually accompanied by an enlarged plasma volume, and therefore indicates an enlarged plasma cholesterol pool size (Lei, 1990b). Al-Othman and Lei (1989) reported that the enlarged plasma volume and cholesterol pool size actually precede the increase in cholesterol concentration. Both of the two

forms of cholesterol, free cholesterol and cholesterol esters, become elevated in the plasma, with the ratio between the two preserved (Lei 1978). Although the etiology of the deficiency-induced hypercholesterolemia is not entirely clear, studies on cholesterol degradation, elimination, and synthesis, as well as on related enzymes and tissue cholesterol content in rats have provided much insight into the mechanisms possibly involved.

Cholesterol Degradation and Elimination Studies

The oxidation of cholesterol to bile acids was measured by Lei (1978) using 26-¹⁴C-labeled cholesterol, and in the same study, the feces were analyzed for the tracer as an estimation of cholesterol excretion. The results revealed that the degradation and elimination of cholesterol is probably not altered by copper deficiency. Allen and Klevay (1978, as cited in Lei, 1990b) also furnished evidence towards this conclusion by measuring biliary excretions collected from a cannulated bile duct as a means of approximating the bile acid synthesis and secretion, and cholesterol elimination. The excretions of bile acid and cholesterol were not seen to diminish in the deficient state.

Lin and Lei (1981) used [4-¹⁴C]cholesterol clearance to measure cholesterol degradation by the two-pool kinetic model. In rats and humans, plasma cholesterol appears to turnover in congruence with the two-pool model. In this model the cholesterol pool from certain tissues equilibrates quickly (QE) with plasma cholesterol, while the pool from other tissues equilibrates much slower (SE). The QE pool is comprised of cholesterol from the plasma, liver, red blood cells, spleen, kidney, lung, and intestine, whereas the SE pool

consists of cholesterol from all the other sources (Lei, 1990b). In this study, copper deficiency was shown to impact the pools by enlarging the QE pool, and decreasing the loss from the QE pool by increasing the half-life of cholesterol. As a note, the QE pool should not be misinterpreted to represent the plasma pool alone.

Cholesterol Synthesis Studies

The results of studies that measured ^{14}C -labeled acetate in incubated rat liver slices (Lei, 1977), and HMG-CoA reductase activity in the rat liver (Yount et al., 1990), and ^3H -labeled water incorporated into various organs (Yount et al., 1991) lend support to the logical explanation that deficiency-induced hypercholesterolemia is due to a persistent amplification of cholesterol synthesis (Lei, 1990b). In the study by Lei (1977) which used ^{14}C -labeled acetate, the rate of hepatic cholesterol synthesis was operationally defined as the amount of the tracer found in digitonin-precipitable sterols (DPS) after a specific incubation period. When the data was standardized for organ and body weight, a significant increase in the cholesterol synthesis was seen in the copper-deficient animals as compared to the copper-adequate animals. In the same study, the researcher also identified enhanced fatty acid synthesis in the deficient rats.

HMG-CoA reductase activity was analyzed in rat hepatocytes as a function of copper status by Yount et al. (1990). The activity of this enzyme is an indicator of cholesterol synthesis because it catalyzes the conversion of HMG-CoA into mevalonate, which is the rate-limiting and committed step in cholesterol synthesis. The liver is the predominant site of cholesterol synthesis in the rat

(Lei, 1990b), and is, thus, the tissue of choice for synthesis predictions in this animal. Yount et al. found that when the animals were not fasted prior to sacrifice, the amount of total and active HMG-CoA reductase was larger in the deficient rats than in the adequate rats. These findings suggest an inflated ability to synthesize cholesterol in the deficient animal.

Cholesterol synthesis was measured *in vivo* by the rate of ³H-water incorporation into DPS (Yount et al., 1989). The labeled DPS in the plasma was also analyzed to determine the amount of newly synthesized cholesterol secreted from the tissues. Although tissue cholesterol synthesis in the deficient animals was only slightly higher than in the adequate animals, the amount of newly synthesized cholesterol that was transferred into the plasma was magnified two-fold in deficiency. The premise that copper-deficient animals transfer more cholesterol from the liver to the plasma was first substantiated by Shao and Lei (1980; as cited in Lei, 1990b). These researchers measured the conversion of [2-¹⁴C]mevalonate into cholesterol in hepatic tissues and found that newly synthesized cholesterol esters are cleared from the liver faster in copper-deficient rats than in copper-adequate rats. This enhanced turnover rate of the liver cholesterol ester pool seemed to coincide with a depressed turnover rate of the serum cholesterol ester pool.

Studies on Related Enzymes

It has been hypothesized that LCAT, which is involved in the transfer process of peripheral cholesterol to the liver via reverse cholesterol transport, may be linked to the hypercholesterolemia of copper deficiency. Although there is

not entire concordance on the finding, LCAT activity has been observed to decline in the plasma of copper-deficient rats (Lau & Klevay, 1981, as cited in Lei, 1990b; Harvey & Allen, 1981). Likewise, lipoprotein lipase, the catalyst for the hydrolysis of VLDL, IDL, and chylomicron triglycerides, has been with discrepancy shown to diminish in the deficient state (Lau & Klevay, 1982; Koo et al., 1988, as cited by Lei, 1990b).

Studies on Tissue Cholesterol Content

A reduction in total cholesterol in the liver following copper deficiency has been documented many times over (Lei, 1990b). In the previously described study in which Lei (1978) used 26-¹⁴C-labeled cholesterol to measure cholesterol synthesis and excretion, liver cholesterol values were also determined. Free and esterified cholesterol were distinguished, and the data demonstrated a proportional depression of both forms of cholesterol in the liver. Since then, this finding has been confirmed using other methods of analysis (Lefevre et al., 1985, as cited in Lei, 1990b). In contrast, uniform data does not exist to suggest that the cholesterol concentration in other tissues, such as the heart and kidney, are altered in congruence with minimized copper intake (Lei, 1990²).

From these mentioned studies emerged a withstanding theory proposed by Lei (1977, 1990b) that liver cholesterol is shifted to the serum pool, making it unavailable for bile acid synthesis and elimination. Low cholesterol levels in the liver are able to up-regulate HMG-CoA reductase (Brown & Goldstein, 1986), prompting stimulated cholesterol synthesis, followed by enhanced

lipoprotein synthesis and secretion. The increased fatty acid synthesis documented in deficiency may provide the capabilities to sustain the enhanced lipoprotein production. A prolonged net efflux of cholesterol from the liver to the plasma may lead to the observed diminished liver cholesterol levels and increased plasma cholesterol levels.

This theory has sustained and been extended to include the findings from subsequent studies on lipoprotein metabolism. The composition and metabolism of the lipoprotein particles have been described to change in copper deficiency, and the change seems to vary in different species (Lei & Carr, 1990). Most of the studies have been conducted on rats, yet there have been a few on humans and rabbits.

In rats, the deficiency-induced rise in plasma cholesterol appears to be localized primarily in the HDL fraction. Although no change has been detected in the percentage of total plasma cholesterol carried in HDL, there is a build up of total and esterified cholesterol in HDL (Lei & Carr, 1990). A significant increase in the absolute amount of LDL cholesterol has also been exhibited, along with an accumulation of LDL triglycerides (Lei et al., 1983). In contrast to HDL, the percentage of total plasma cholesterol carried in LDL is greater in the deficient rat compared to the control, and the percentage in VLDL is reduced (Lei et al., 1983).

As would be expected, the enlarged lipid content of HDL and LDL is accompanied by an elevation in total protein content in each of the fractions (Lei et al., 1983). A proliferation in apo E has been identified to account for

the protein increase in HDL. There is also a tendency for apo A_I to increase in copper deficiency, but the increase is thought to be smaller than that of apo E (Lei & Carr, 1990). It has been suggested that a greater number of apo E-rich HDL particles may result from copper deficiency. This case has been supported by findings that most of the increase in cholesterol and protein content of HDL is in the apo E-rich subfraction (Crowell & Lei, 1989). In addition, composition data that revealed a proportional increase in HDL lipids to protein implies that the number of HDL particles is increased, while the size remains roughly the same in deficiency (Lei et al., 1983; Lei & Carr, 1990).

The removal rate of HDL subfractions from the plasma has been shown to increase in copper-deficient rats (Carr & Lei, 1989), with virtually all of the HDL cholesterol esters being delivered to the liver (Carr & Lei, 1990). The specific uptake system of apo E-rich HDL is thought to be distinct from the LDL or apo E receptor, and the binding of the apo E-rich HDL to the sites has been demonstrated to be greater in copper-deficient rats than in copper-adequate rats (Hassel et al., 1988).

The information rendered from these studies on lipoprotein metabolism in copper deficiency has been appended to the prevailing theory by Lei (1977, 1990b) for hypercholesterolemia. Additional to Lei's theory, it is hypothesized that an expanded plasma HDL pool with enlarged cholesterol content delivers increased amounts of cholesterol to the liver to support the enhanced production of lipoproteins that transfer cholesterol from the liver to the plasma in copper deficiency. Carr and Lei (1990) further hypothesized that the

hypercholesterolemia could be due to an imbalance between influx and efflux of the HDL free and esterified cholesterol.

The results of the few human and rabbit studies (Lei & Carr, 1990) implied that the alterations in lipoprotein composition as a result of copper deficiency may differ from species to species, especially depending on the lipoprotein class in which the bulk of cholesterol is transported. The human and rabbit, both of which carry the majority of cholesterol in LDL, appear to primarily localize the excess cholesterol resulting from copper deficiency in the LDL fraction. The protein, as well as cholesterol content have been described to increase in the LDL of these species during copper deficiency.

MATERIALS AND METHODS

Animals and Design - Study 1

Twenty-three Golden-Syrian male hamsters were obtained from SASCO (Omaha, NE 68101) at three weeks of age. Upon arrival, each hamster weighed between 42 and 56 grams, and was randomly assigned to one of two dietary conditions: copper-deficient (12 animals) or copper-adequate (11 animals). Initially the animals were housed three in a cage. But after a hamster from each condition was injured in the large grating of the group-cages, the animals were transferred to individual suspended stainless steel wire cages with smaller grating. They were separated at the end of the second week and the two injured hamsters were excluded from the experiment. The animal facility was kept on a 12 hour light/12 hour darkness cycle and was maintained at 22°C. The specific diet was provided ad libitum, as was distilled-demineralized water (distilled water passed through an ion-exchange resin; apparatus purchased from Barnstead Co., Sybron Corp., Boston, MA). Weight gain and food intake were examined at various times throughout the study.

After eight and a half weeks of dietary treatment, the hamsters were fasted for 24 hours and then anesthetized with carbon dioxide followed by diethyl ether. The heart was punctured and blood was collected into a syringe that was prepared beforehand with ethylenediamine tetracetic acid, EDTA (~1 mg/ml blood). The percentage of blood hematocrit was determined and the plasma was separated by centrifugation. The heart, liver, kidneys and spleen were

excised, weighed, and immediately stored in a freezer. Plasma volume was calculated by subtracting the total erythrocyte volume from the total blood volume as described in Vander et al. (1985). The blood volume was computed from the average percent volume of body weight, and the erythrocyte volume was determined by multiplying the hematocrit value of each animal by the blood volume.

Animals and Design - Study 2

In this experiment, 14 female Golden-Syrian hamsters (purchased from SASCO, Omaha, NE 68101) were randomly divided into two dietary treatments; 8 animals were fed a copper deficient diet, and 6 animals were fed a copper adequate diet. The hamsters were three weeks old upon arrival and weighed between 42 and 64 grams. They were housed individually in identical suspended stainless steel wire cages and the room was kept on a 12 hour light/12 hour darkness cycle at 22°C. The respective diet and distilled-demineralized water were provided ad libitum. The weight gain of each animal was assessed weekly and their food intake was recorded in two week periods.

At the end of five and a half weeks on the experimental diet, each hamster was anesthetized with carbon dioxide followed by diethyl ether. Using the same procedures as in Study 1, blood was removed from the heart, the hematocrit was evaluated, and the plasma was isolated. The heart, liver, kidneys and spleen were removed, weighed and frozen. Plasma volume was determined as described above.

Experimental Diet

The animals in both studies were fed a diet based on the nutrient requirements for laboratory hamsters defined by The National Research Council (1978), excluding any copper supplementation. This exact diet was used for the deficient treatment and contained 0.54 mg Cu/kg diet, as measured by atomic absorption spectrophotometry. The diet used for the copper-adequate treatment was fortified with copper carbonate and contained 6.40 mg Cu/kg diet. The composition of the diet for each treatment is outlined in Table 4.

Separation of Plasma Lipoproteins

After all the blood samples were obtained, the plasma was separated from the red and white blood cells and the platelets by centrifugation for 20 minutes at 5000 x g at 15°C. The plasma was then carefully removed from the top of each centrifuge tube and plasma from three to four animals were combined. The combining process of Study 1 provided three samples from each treatment, and that of Study 2 provided two samples from each treatment. In Study 2, the animals were not fasted the night before being sacrificed, and therefore, to avoid contaminating the VLDL fractions, the chylomicrons needed to be removed before the other lipoproteins could be separated. The chylomicrons were removed by gently placing overlayer buffer on top of the plasma samples and spinning them in a Beckman Model L8-80 ultracentrifuge (Fullerton, CA) for 30 minutes at 10,000 g at 15°C. After spinning, the top layer that contained the chylomicrons was removed.

Table 4. Diet Composition - Study 1 and 2.

Ingredient	Copper Deficient	Copper Adequate
Percent Composition		
Casein	24.0	24.0
Fiber Cellulose	6.0	6.0
Glucose Monohydrate	59.6	58.6
Corn Oil	5.0	5.0
DL-Methionine	0.3	0.3
AIN Mineral Mix ¹	4.0	4.0
Vitamin Mix	1.0	1.0
Choline Bitartrate	0.1	0.1
Cupric Carbonate Mix (1.05 g/kg)	—	1.0
Dietary Copper (ppm) ²	0.54	6.40

¹Contains all minerals excluding copper.

²Determined by atomic absorption spectrophotometry.

The plasma lipoproteins were separated using the method described by Rudel et al. (1974). First the lipoproteins were floated into a buffer solution. This was done by transferring the combined, chylomicron-free, plasma samples into ultracentrifuge tubes and raising the density to d 1.225 with the addition of solid KBr (.3517 g/ml plasma). Five ml of overlayer buffer (d 1.225) was gently placed on top to form a clear, distinct layer above the plasma. The tubes were balanced by adding drops of overlayer buffer and spun in a swinging-bucket Ti-70 rotor in a Beckman model L8-80 ultracentrifuge (Fullerton, CA) at 40,000 g for 24 hours at 15°C.

After ultracentrifugation, the top 3 to 4 ml of the overlayer buffer, which then contained the lipoproteins, were transferred into twist-capped tubes. The contents of each tube were then individually passed through an agarose bead gel column (2.5 x 90 cm; Bio-Rad, Richmond, CA). The apparatus was stored and used in a cold room. The column was well-packed and an elution buffer (0.01% EDTA, 0.02% Na-Azide, 0.15% NaCl, pH 7.4) was allowed to equilibrate for 12 hours. The lipoproteins were injected quantitatively and eluted at 20 ml/hour. The eluate was collected in fractions by a LKB Model 2111 fraction collector (LKB instruments Inc., Rockville, MD) and the protein content was detected and recorded at 280 nm. The fractions to be combined as specific lipoprotein types were determined by the peaks in the recorded chart. The first lipoprotein type to elute was very low density (VLDL), followed by low density (LDL), and then high density (HDL), which correspond to peaks 1, 2, and 3, respectively. The specific lipoprotein types will now be referred to as

lipoprotein fractions, not to be confused with the eluted fractions that were combined to make up these specific lipoprotein fractions.

Concentration of Lipoprotein Fractions

The specific lipoprotein fractions were standardized in volume and placed in separate nalgene bottles. In Study 1, a protein assay was performed before concentrating the fractions. In Study 2, two ml of each sample was set aside for the protein assay, and the remaining sample was concentrated shortly after elution. The pre-concentration protein assays were used along with post-concentration assays to determine the percent recovery from the concentration process. The percent recovery values were accounted for in the cholesterol and triglyceride analysis as a correction factor. Each sample was concentrated to 2 to 3 ml by ultracentrifugation using YM-30 filters in Amicon stirring-cells (Amino Corp., Danvers, MD) and stored in screw-cap tubes for subsequent protein, cholesterol, and triglyceride analyses.

Protein Analysis of Lipoprotein Fractions

The spectrometric method of Lowry et al. (1951) was used to quantitatively analyze the protein content of the lipoprotein fractions. In the Lowry assay, copper is added to the samples and binds to the protein, simultaneously reducing a phosphotungstic-phosphomolybdic agent that can be measured colorimetrically. Specifically, distilled water and 2.5 ml of a reagent (2% Na_2CO_3 , 2% potassium tartrate, and 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were added to a determined portion of the sample. A 1.0 N Folin reagent was then added and mixed, and the solution was let to stand for 30 minutes. In the pre- and post-

concentration VLDL samples of Study 2, 20 μ l of triton was added to clear the opaque color caused by the large triglyceride content. As a control, the same amount was also added to the standards for these assays only. The solutions were read against a blank at 750 nm with visible light by a Beckman Model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

The light-absorption measurement is related to the protein concentration by Beer's Law, $A=Ebc$, with E being the molar absorptivity and b being the width of the spectrometric cuvette. Graded levels of protein standards (0, 5, 10, 20, 30, 40, 50 mg) were used to determine an index of molar absorptivity by plotting a standard curve. Bovine albumin (Sigma Chemical Co., St. Louis, MO) was used as the protein standard and the same reagents were added to the set of standards as to the samples. The concentration of protein in each plasma lipoprotein fraction was backcalculated from the concentration in the cuvette by using the volumes noted in the previously described separation technique.

Cholesterol Analysis of Lipoprotein Fractions

The cholesterol content of each lipoprotein fraction was quantitatively analyzed by using an enzymatic kit purchased from Boehringer Mannheim (Indianapolis, IN). The enzymatic reagent included in the kit contained cholesterol esterase, which acts to split all cholesteryl esters into free cholesterol and fatty acids. The free cholesterol in the sample, present prior to and derived from the hydrolysis by cholesterol esterase, was oxidized to cholest-4-en-3-one with the release of hydrogen peroxide. The hydrogen

peroxide was reacted with phenol and 4-aminophenazone to form an imine dye with an intensity directly proportional to the original cholesterol concentration. The color was measured photometrically at 500 nm by the Beckman Model 25 spectrometer described in the previous section on protein analysis. A standard curve was constructed by photometrically measuring the color produced by reacting the enzymatic reagent with certified standards of cholesterol (50, 100, 150, 200, 300, 400 mg/dl; Boehringer Mannheim). Similar to the protein analysis, the concentration of cholesterol in each plasma lipoprotein fraction was backcalculated.

Triglyceride Analysis of Lipoprotein Fractions

To determine the amount of triglyceride in the lipoprotein fractions, an enzymatic kit was used (Sigma; St. Louis, MO). The first reaction catalyzed by the enzymatic reagent of the kit was the hydrolysis of triglycerides to glycerol and fatty acids by lipoprotein lipase. Glycerol was then phosphorylated via ATP, and the product reacted with NAD to render NADH. In the presence of diaphorase, the NADH was returned to its oxidized state by the reduction of 2-(p-iodophenyl)-3-p-nitro-phenyl-5-phenyltetrazolium chloride (INT) to Formazan (INTH). Formazan is colored and provides a measurement of triglyceride concentration by colorspectroscopy. The resulting color from the interaction between the triglycerides and the enzymatic reagent was read at 500 nm using a Beckman Model 25 spectrophotometer. In order to determine the triglyceride concentrations, an absorption factor was computed using the absorption reading of a certified standard that was also reacted with the

enzymatic reagent. The concentration in the plasma fractions were backcalculated.

Mineral Analysis of the Liver

The copper content of the liver was analyzed by flame atomic absorption spectrophotometry after digestion with nitric acid. To digest the tissue, 0.5 to 1 gram samples were first dried at 80°C for 4 days. The samples were then allowed to cool 24 hours in a desiccator before a dry weight was measured. The dried samples were packed in an ice bath, and under a hood, 3 ml of concentrated nitric acid were added to digest the tissue. After 24 hours the contents were vortex-mixed and the tubes were placed in a hot water bath (95-100°C) for 3 hours, also maintained under a hood. The samples were allowed to cool and then diluted to 5 ml and analyzed for copper using a Hitachi 180-70 Polarized Zeeman atomic absorption spectrometer (Mountain View, CA).

RESULTS

Study 1

In the first experiment, a significant difference in mean body weight was recorded as early as the second week on the dietary treatment. As illustrated in Figure 1, the animals fed a copper-deficient diet weighed on the average less than the animals fed an adequate diet, and the difference extended throughout the duration of the study. Only after the sixth week was the amount of diet consumed significantly larger by the animals on the adequate diet than by those on the deficient diet. The effects of treatment and duration on food intake are displayed in Figure 2. Two sample independent t-tests were used to compare the means between treatments for body weight and food intake, and significant differences are indicated by asterisks in both Figure 1 and 2.

Of the usual signs indicating copper deficiency, body weight and hematocrit were significantly reduced in the hamsters fed the copper-deficient diet. In addition, the average plasma volume of these animals was evidently enlarged, along with the liver to body weight ratio. The analysis of the liver copper content indicated that on the average, the deficient animals had lower copper stores than the adequate animals. There was actually a 35% decrease in liver copper as a result of the deficient treatment, which was significant. The differences in body and organ weights, as well as hematocrit and plasma volumes, and liver copper content between experimental conditions were evaluated using one-way ANOVA tests, and the values are shown in Table 5.

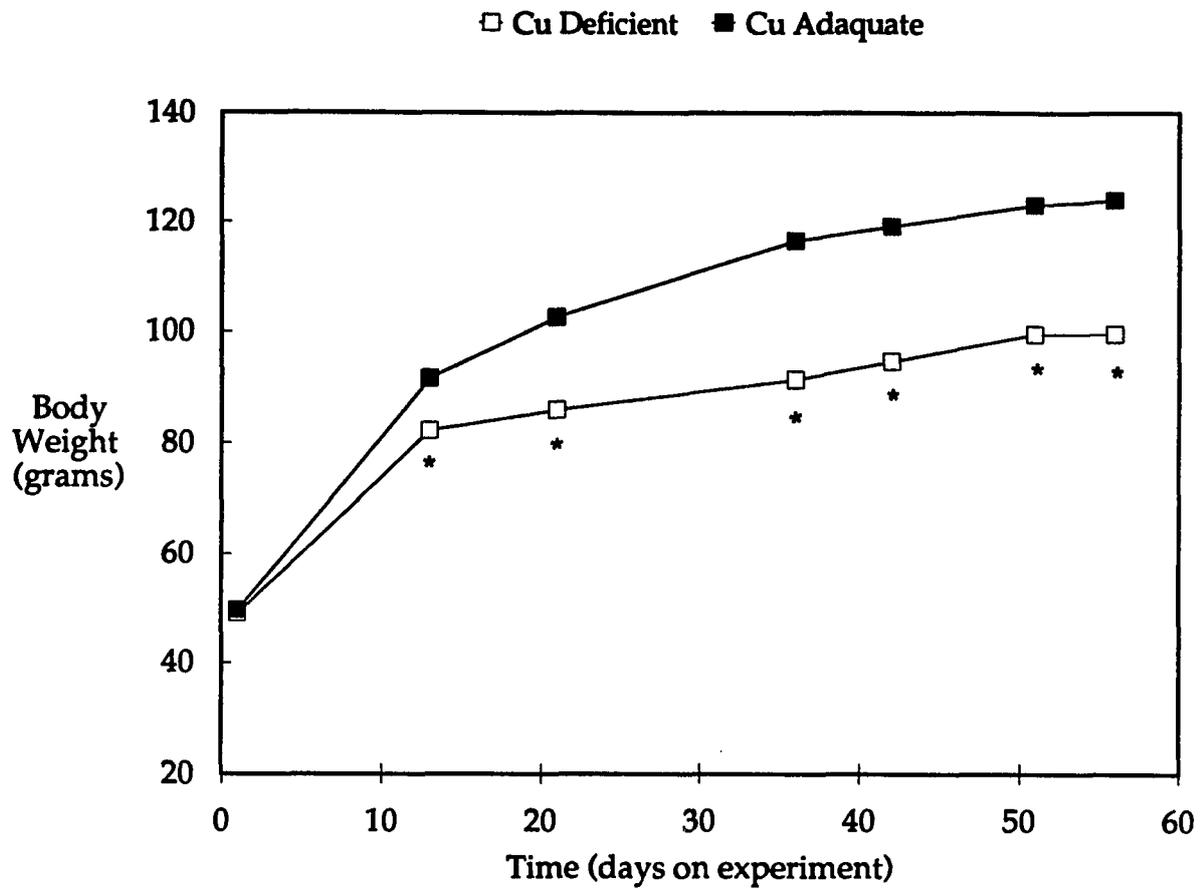


Figure 1. The Influence of Dietary Copper and Time on Body Weight of Male Hamsters - Study 1. Data points represent mean weights, and significant differences between treatments, determined by two-sample independent t-tests, are denoted by * ($p < 0.01$).

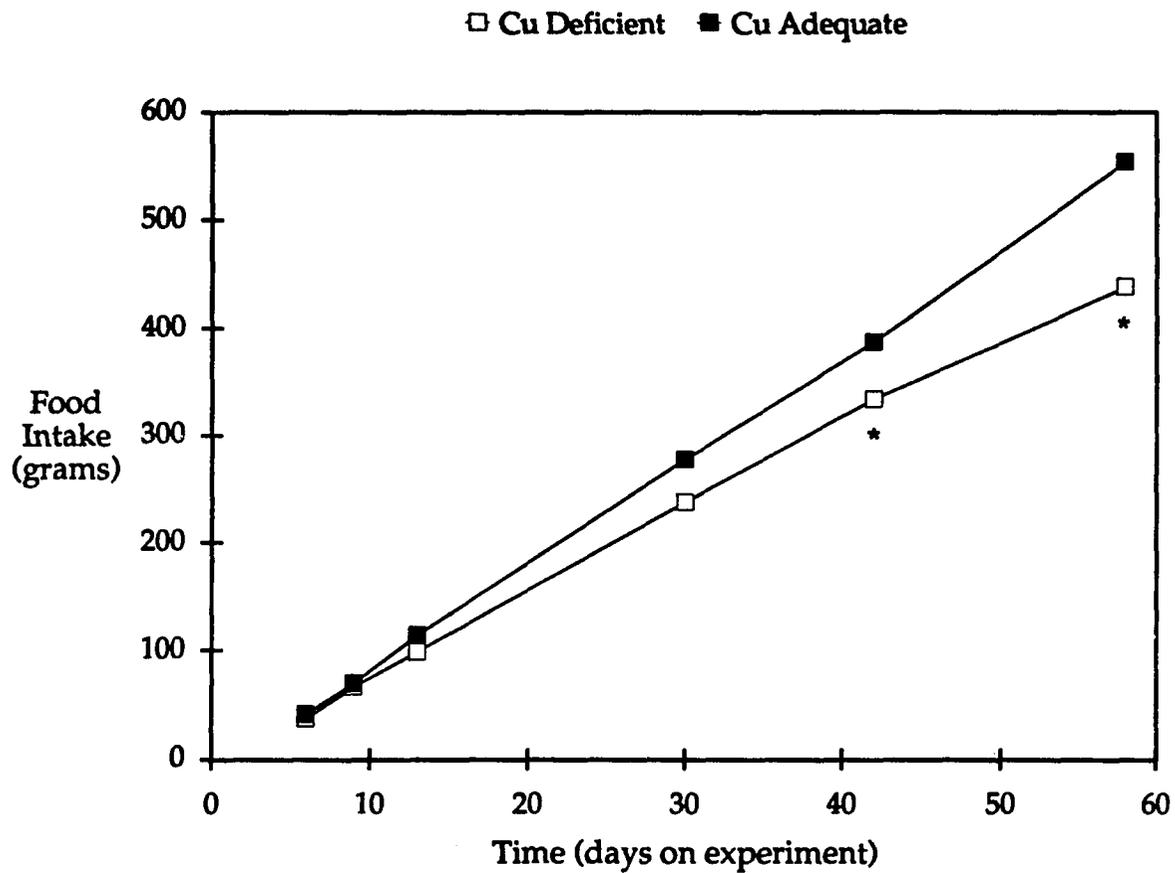


Figure 2. The Influence of Dietary Copper and Time on Food Intake by Male Hamsters - Study 1. Data points represent the average cumulative amount of diet consumed, and significant differences between treatments, determined by two-sample independent t-tests, are denoted by * ($p < 0.01$).

Table 5. The Effect of Copper Deficiency on Body and Organ Weights, Hematocrit, Plasma Volume, and Liver Copper Content in Male Hamsters - Study 1¹

	Copper Adequate	Copper Deficient	p-value ²
Body Weight (g)	114 ± 4	91 ± 2	<0.001
Liver Weight (g/100 g body wt)	3.33 ± 0.06	3.76 ± 0.11	<0.004
Heart Weight (g/100 g body wt)	0.42 ± 0.02	0.45 ± 0.01	NS
Kidney Weight (g/100 g body wt)	0.90 ± 0.04	1.07 ± 0.04	<0.006
Spleen Weight (g/100 g body wt)	0.11 ± 0.00	0.15 ± 0.01	<0.006
Hematocrit (% packed cell volume)	53.1 ± 0.4	47.0 ± 0.8	<0.001
Plasma Volume (ml/100 g body wt)	3.28 ± 0.03	3.71 ± 0.05	<0.001
Liver Copper Content (µg/g wet weight)	7.43 ± 0.11	4.84 ± 0.23	<0.001

¹Means ± SEM.

²One Way ANOVA; NS = not significant.

The hematocrit was reduced by 11% in the deficient animals and the plasma volume was enlarged by 13% as compared to the control animals.

Previously reported changes in cholesterol concentrations in the various lipoprotein fractions as a result of a copper-deficient diet (Al-Othman, 1989) did not manifest in this study. Likewise, differences in triglyceride and in protein concentrations were not observed between the copper-deficient and adequate treatments. Even when the lipoprotein data were expressed as the amount present in the vascular pool corrected for body weight, there was no deviation in cholesterol, triglyceride, or protein between the copper-deficient and adequate groups. In fact, except for the HDL particles, the cholesterol plasma pool sizes showed a very slight tendency to be higher in the copper-adequate animals. The concentrations and plasma pool sizes of cholesterol, triglyceride, and protein in the specific lipoprotein fractions are presented in Table 6 and 7, respectively. The percentage of total plasma cholesterol carried by the lipoprotein fractions appeared to be altered by dietary treatments, but not significantly. The percent of cholesterol in VLDL decreased by 6%, increased in HDL by 6%, and remained unchanged in LDL. The percent values and standard errors are listed in Table 8.

Table 6. The Effect of Copper Deficiency on Cholesterol, Triglyceride and Protein Concentration in Plasma Lipoprotein Fractions of Male Hamsters - Study 1¹

Fraction	Copper Adequate	Copper Deficient	p-value ²
Plasma Concentration (mg/dl plasma)			
Cholesterol			
VLDL	21.9 ± 7.0	10.7 ± 4.5	NS
LDL	32.2 ± 2.3	26.5 ± 4.3	NS
HDL	74.0 ± 3.9	66.4 ± 0.6	NS
Triglyceride			
VLDL	100.2 ± 42.9	41.4 ± 13.6	NS
LDL	16.8 ± 5.5	14.2 ± 4.2	NS
HDL	16.3 ± 3.7	21.5 ± 7.4	NS
Protein			
VLDL	19.0 ± 4.7	12.3 ± 4.5	NS
LDL	26.5 ± 2.0	23.7 ± 1.5	NS
HDL	186.7 ± 6.2	173.2 ± 2.2	NS

¹Means ± SEM.

²One Way ANOVA; NS = not significant.

Table 7. The Effect of Copper Deficiency on the Plasma Pool Size of Cholesterol, Triglyceride and Protein in Plasma Lipoprotein Fractions of Male Hamsters - Study 1¹

Fraction	Copper Adequate	Copper Deficient	p-value ²
Plasma Pool Size (mg/100 g body weight)			
Cholesterol			
VLDL	0.72 ± 0.24	0.34 ± 0.16	NS
LDL	1.07 ± 0.08	0.97 ± 0.14	NS
HDL	2.45 ± 0.15	2.44 ± 0.08	NS
Triglyceride			
VLDL	3.33 ± 1.40	1.52 ± 0.50	NS
LDL	0.56 ± 0.19	0.52 ± 0.14	NS
HDL	0.54 ± 0.12	0.78 ± 0.25	NS
Protein			
VLDL	0.63 ± 0.16	0.45 ± 0.16	NS
LDL	0.88 ± 0.07	0.87 ± 0.04	NS
HDL	6.17 ± 0.25	6.37 ± 0.21	NS

¹Means ± SEM.

²One Way ANOVA; NS = not significant.

Table 8. Percentages of Total Plasma Cholesterol Carried by the Respective Lipoprotein Fractions in Male and Female Hamsters - Studies 1, 2, and 1 and 2 Collapsed¹

Fraction	Copper Adequate	Copper Deficient	Copper Effect p-value ²
Percent Total Plasma Cholesterol			
Study 1			
VLDL	16.3 ± 3.7	10.0 ± 3.7	NS
LDL	25.3 ± 0.8	25.3 ± 3.1	NS
HDL	58.4 ± 2.9	64.7 ± 4.4	NS
Study 2			
VLDL	7.9 ± 0.5	5.1 ± 2.1	NS
LDL	26.7 ± 0.9	31.1 ± 3.2	NS
HDL	65.4 ± 0.4	63.8 ± 1.1	NS
Study 1 and 2			
VLDL	13.0 ± 2.9	8.1 ± 2.4	NS
LDL	25.8 ± 0.7	27.6 ± 2.4	NS
HDL	61.2 ± 2.4	64.3 ± 2.5	NS

¹Percent ± SEM.

²One Way ANOVA (Study 1, Study 2); Two Way ANOVA (combined result from Study 1 and 2; copper effect, DF=1; gender effect, DF=1, NS; interaction, DF=1, NS); NS = not significant.

Study 2

In this experiment, the body weights between the copper-deficient and adequate groups did not differ significantly at any point during the treatment period. This is depicted in Figure 3. As seen in Figure 4, there was an unequal amount of food consumed at the end of the fourth and fifth week of dietary treatment. The deficient animals consumed less diet than the adequate animals in these weeks.

Similar to the results of Study 1, the hematocrit was significantly lower and the plasma volume was elevated in the deficient hamsters compared to the adequate hamsters. The hematocrit decreased by 11% and the plasma volume enlarged by 11% compared to the controls. In this study, no difference was seen in body, heart, kidney or spleen weight between the two experimental conditions. The mineral analysis of the liver indicated a substantial difference in copper content between the two treatments. Similar to the finding in Study 1, the deficient group had much lower stores than the adequate group; there was a 71% decrease due to the deficient diet as compared to the adequate diet. One-way ANOVA tests were used to analyze the differences and the values are listed in Table 9.

In contrast to Study 1, the cholesterol in the HDL fraction of the deficient treatment differed significantly from that of the adequate treatment. Surprisingly, the deficient group contained lower concentrations than the adequate group. When the data was converted to the pool size in the plasma

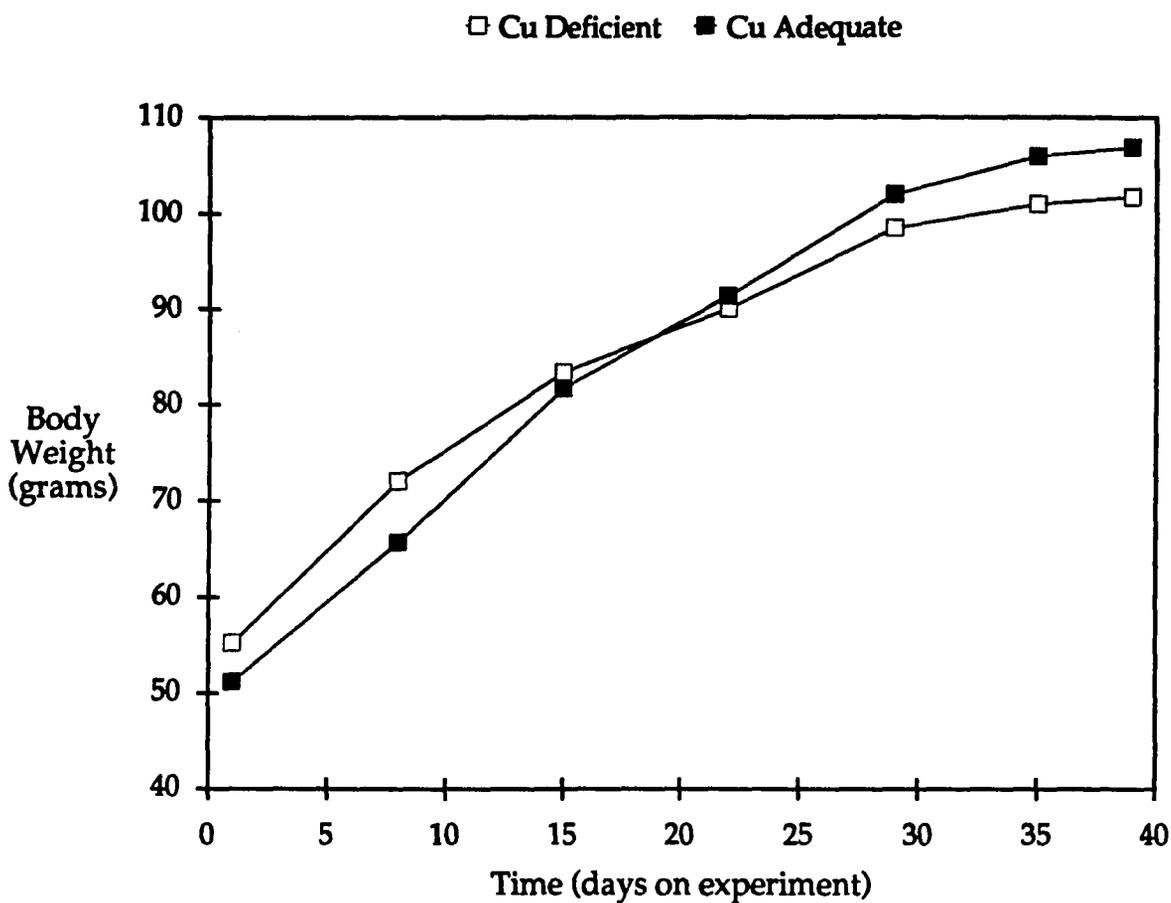


Figure 3. The Influence of Dietary Copper and Time on Body Weight of Female Hamsters - Study 2. Data points represent mean weights, and two-sample independent t-tests indicated no significant differences between treatments.

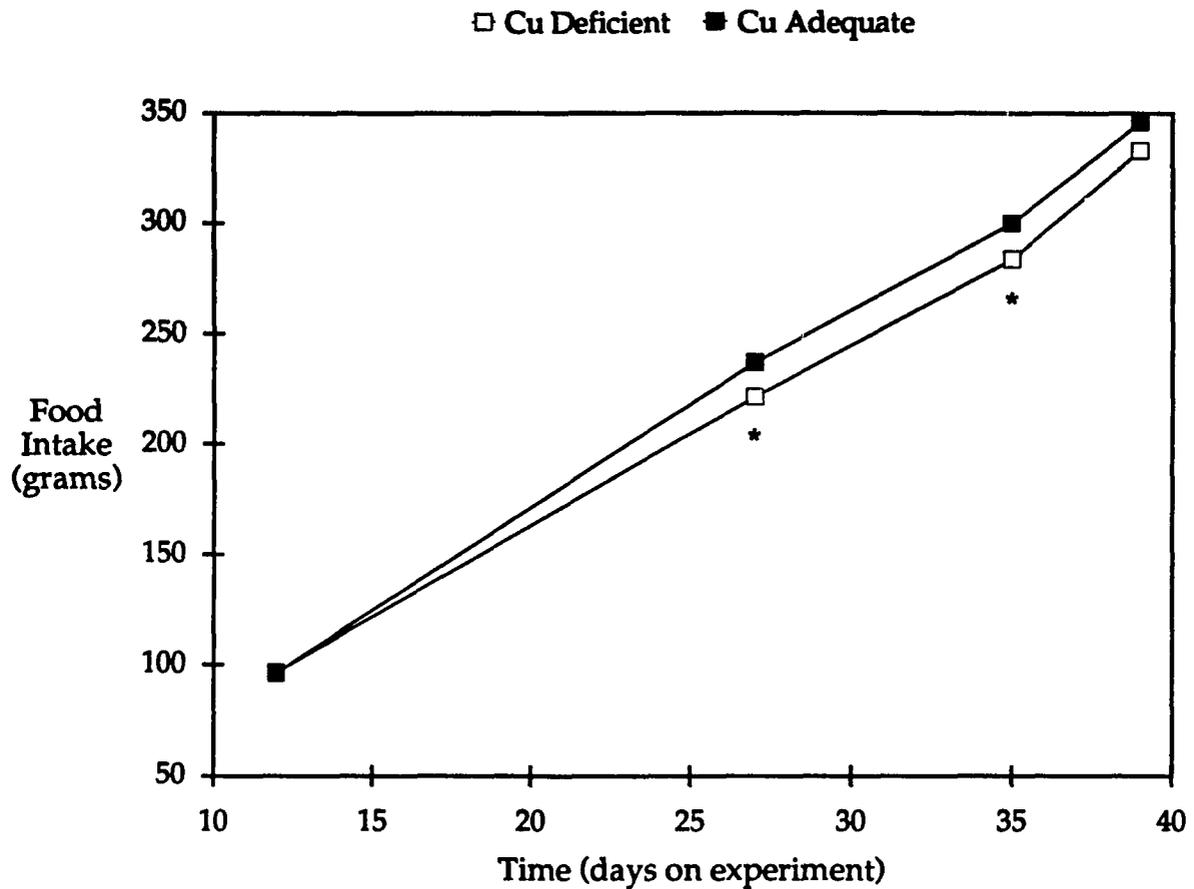


Figure 4. The Influence of Dietary Copper and Time on Food Intake by Female Hamsters - Study 2. Data points represent the average cumulative amount of diet consumed, and significant differences between treatments, determined by two-sample t-test, are denoted by * ($p < 0.01$).

Table 9. The Effect of Copper Deficiency on Body and Organ Weights, Hematocrit, Plasma Volume, and Liver Copper Content in Female Hamsters - Study 2¹

	Copper Adequate	Copper Deficient	p-value ²
Body Weight (g)	106 ± 2	102 ± 3	NS
Liver Weight (g/100 g body wt)	5.22 ± 0.16	4.65 ± 0.13	<0.017
Heart Weight (g/100 g body wt)	0.42 ± 0.01	0.40 ± 0.01	NS
Kidney Weight (g/100 g body wt)	1.04 ± 0.05	1.03 ± 0.03	NS
Spleen Weight (g/100 g body wt)	0.14 ± 0.01	0.16 ± 0.01	NS
Hematocrit (% packed cell volume)	50.1 ± 0.3	44.6 ± 0.4	<0.001
Plasma Volume (ml/100 g body wt)	3.50 ± 0.02	3.88 ± 0.03	<0.001
Liver Copper Content (µg/g wet weight)	5.20 ± 0.23	1.46 ± 0.15	<0.001

¹Means ± SEM.

²One Way ANOVA; NS = not significant.

of a hamster of 100 grams body weight, the HDL cholesterol pool size remained significantly larger in the adequate treatment as compared to the deficient treatment. The differences in concentration and pool sizes were evaluated using one-way ANOVA tests and the values are listed in Tables 10 and 11, respectively. The percentage of total plasma cholesterol carried by the respective lipoprotein fractions tended to change due to deficiency; however, these changes were not significant. Table 8 lists the percentage values. The cholesterol carried by VLDL was reduced by about 3% with deficiency; the LDL-carried cholesterol increased by 4%, and the percent carried by HDL remained virtually unaltered. When the percentages of lipoprotein-carried cholesterol from both Study 1 and 2 were collapsed, the percentage alterations remained insignificant.

Table 10. The Effect of Copper Deficiency on Cholesterol, Triglyceride and Protein Concentration in Plasma Lipoprotein Fractions of Female Hamsters - Study 2¹

Fraction	Copper Adequate	Copper Deficient	p-value ²
Plasma Concentration (mg/dl plasma)			
Cholesterol			
VLDL	10.8 ± 1.3	4.7 ± 1.8	NS
LDL	36.1 ± 1.0	28.8 ± 3.3	NS
HDL	88.7 ± 5.9	59.1 ± 0.3	<0.038
Triglyceride			
VLDL	89.8 ± 3.5	82.3 ± 0.3	NS
LDL	9.7 ± 0.3	8.8 ± 1.0	NS
HDL	16.5 ± 5.3	5.9 ± 0.9	NS
Protein			
VLDL	9.3 ± 0.9	4.9 ± 0.8	NS
LDL	36.1 ± 0.9	32.9 ± 3.5	NS
HDL	193.7 ± 19.0	147.1 ± 0.6	NS

¹Means ± SEM.

²One Way ANOVA; NS = not significant.

Table 11. The Effect of Copper Deficiency on the Plasma Pool Size of Cholesterol, Triglyceride and Protein in Plasma Lipoprotein Fractions of Female Hamsters - Study 2¹

Fraction	Copper Adequate	Copper Deficient	p-value ²
Plasma Pool Size (mg/100 g body weight)			
Cholesterol			
VLDL	0.38 ± 0.04	0.18 ± 0.07	NS
LDL	1.26 ± 0.02	1.12 ± 0.12	NS
HDL	3.10 ± 0.18	2.30 ± 0.02	<0.008
Triglyceride			
VLDL	3.14 ± 0.15	3.20 ± 0.01	NS
LDL	0.34 ± 0.01	0.34 ± 0.04	NS
HDL	0.58 ± 0.18	0.23 ± 0.04	NS
Protein			
VLDL	0.32 ± 0.03	0.19 ± 0.03	NS
LDL	1.26 ± 0.01	1.28 ± 0.13	NS
HDL	6.76 ± 0.61	5.71 ± 0.01	NS

¹Means ± SEM.

²One Way ANOVA; NS = not significant.

DISCUSSION

Study 1

A number of abnormalities resulting from low copper intake are well-established and are used as the criteria for deficiency in the laboratory animal. Of these symptoms, suppressed growth, reduced hematocrit, diminished hepatic copper content, and enlarged plasma volume were all observed in the hamsters in this study. However, an inflated heart to body weight ratio was not demonstrated. In addition, the decreases in hematocrit and liver copper content were a moderate 11% and 35%, respectively, and the increase in the plasma volume was merely 13%. These values indicate only marginal deficiency in these animals. In fact, the percent deviation from the adequate group are even lower than that documented for marginally deficient rats in a previous experiment by Al-Othman (1989). Al-Othman's study was designed to measure time course developments in marginally and severely deficient rats. The results indicated that hematocrit, liver copper content, and plasma volume varied in the expected direction by 18%, 50%, and 23%, respectively, in the marginal animals relative to the adequate animals. By comparison, it appears that the hamsters in the present study were at the most, mildly copper-deficient.

If this is actually the case, it is reasonable that previously reported increases in cholesterol concentration and pool sizes in the various lipoprotein fraction did not occur in this study. An insufficient depletion of copper may be the explanation for the overall absence of compositional alterations (i.e. protein,

triglyceride, as well as cholesterol content) in the lipoprotein fractions in these animals. However, despite only mild deficiency, there was a slight shift in the percent of total plasma cholesterol carried in each of the lipoprotein classes. Similar to the findings of copper depletion studies on rats (Lei et al., 1983), the percentage of total plasma cholesterol carried by VLDL was reduced by 6% in the deficient hamsters. The changes in percentage of total plasma cholesterol in LDL was nonexistent, and was increased by 6% in HDL. In the former studies using rats, a substantial percent increase of plasma cholesterol carried by LDL was observed. The variance between the findings in copper-deficient rat LDL and hamster LDL may be linked to the insufficient copper depletion in the hamsters of this study, or to the difference in species. The results of Study 2 suggest the former.

It is well founded that copper deficiency leads to hypercholesterolemia in many species. Yet, there have been experiments that failed to induce a rise in plasma cholesterol. Although the idea that the severity of deficiency mandates the increase in cholesterol is a likely explanation for the unsuccessful results in this study, other justifications have been given for the failures of previous studies. Lei (1990a) argued that when an animal is in a rapid growth period, large quantities of cholesterol are used for new tissues formation. The demand for cholesterol may be so great that the cholesterol plasma pool may be utilized as fast as copper deficiency induces cholesterol increases. That is, the liver may be producing more cholesterol, but the young animals are also depositing more. Therefore, an increase in plasma cholesterol may not manifest. This is a valid theory; however, it is not

necessarily an interpretation for the results of the present study because the hamsters were sacrificed when weight gain and, hence, growth began to plateau. A residual cholesterol-deposition effect may play a role, but that is difficult to assume.

A more plausible justification for the lack of cholesterol amplification may lie in the postulation by Prohaska et al. (1985, as cited in Lei, 1990a). These researchers suggested that the age, or stage of growth when deficiency commences may be an important factor in the development of hypercholesterolemia. Because an older animal may have the opportunity to accumulate larger copper stores than a younger animal, especially in the liver, the induction of a deficient state that is sufficient to inflate plasma cholesterol may be more difficult, or impossible in the older animal. This explanation seems to be appropriate for the present study because subsequent experiments conducted in our lab have determined that hypercholesterolemia can be induced in hamsters when they are placed on a copper-deficient diet from two weeks of age. In contrast, three week old hamsters were used in both Study 1 and 2.

Study 2

Although gender is not thought to influence the effects of copper deficiency on cholesterol metabolism, the premise is based on only a few studies which demonstrated hypercholesterolemia in female rats and mice. The present experiment was intended to elucidate lipoprotein alterations in the female hamster in response to copper deficiency. However, the results were similar

to those of Study 1, inconclusive. The copper-deprived animals showed reduced hematocrit, diminished hepatic copper content, and a tendency for suppressed growth that was correspondingly 13%, 71%, and 4% below the adequate animals. Also, the plasma volume of the treatment group was enlarged by 11% over the control. In the study by Al-Othman (1989), the hematocrit of the severely deficient rats was 44% lower than that of the rats fed an adequate amount of copper, and the hematocrit of the marginally deficient rats was 18% lower. Hematocrit is the classical criteria for copper deficiency, and the hematocrit of the hamsters in the present study is clearly more similar to that which would be expected from a marginally deficient animal. Except for the reduced liver copper content, which fell between the range that had been documented for marginal (50%) and severe deficiency (82%), all the other quantitated symptoms indicate only a very mild case of deficiency in the treatment animals, as in Study 1.

The estrogen-copper relationship via ceruloplasmin may be responsible for the greater depletion in liver copper concentration in the mildly deficient female hamsters of this study, as compared to the mildly deficient male hamsters of Study 2, but this is just speculation. The differences in the kidney and spleen weight between Study 1 and 2 exemplify a claim by Lei (1990a) that data on the effects of copper deficiency on these organs have been inconsistent.

A finding that was completely unexpected in this experiment was a decrease in the HDL cholesterol concentrations. The rationale for this observation may be that the small reduction in hematocrit and enlargement in plasma volume

may have lead to a dilution of these lipoprotein particles, resulting in the appearance of a reduction in HDL cholesterol. The specific dilution of cholesterol in this lipoprotein may reflect the fact that it is the primary carrier of cholesterol in the hamster. A dilution effect may also account for the tendency in Study 1 for cholesterol, protein, and triglyceride concentrations in all lipoproteins to be slightly lower in the treatment animals.

The relationship of the observed decreases in HDL cholesterol to the established differences between male and female HDL levels is uncertain. Estrogen has been accredited with increasing HDL by stimulating apo A_I protein synthesis, leading to elevated levels of HDL in females as compared to males (Shaefer et al., 1982). The naturally large HDL levels in copper-adequate females, and the link between estrogen and copper (mentioned in the literature review of this paper) may have contributed to an appearance of a decline in HDL cholesterol concentrations.

Actually, because of the expanded plasma volume seen in deficiency, it is more appropriate to describe alterations in lipoproteins by plasma pool sizes than by concentrations. Even after this conversion, the HDL cholesterol pool size was maintained to be diminished in the deficient group. Being that a change in HDL protein pool size did not correspond to the reduction in cholesterol pool size, it is implied that the number of HDL particles did not decrease in the treatment animals. Overall, there seemed to be a shift of total plasma cholesterol to the LDL particle. The percent of total cholesterol in the adequate animals was altered by 3% in VLDL, 4% in LDL, and 1% in HDL. The percent of total cholesterol carried by the LDL in the deficient animals

showed a much greater increase than indicated in Study 1, and the percent carried by VLDL was decreased. The HDL fractions remained virtually equal.

In a study by Reiser et al.(1987, as cited in Lei, 1990a) in which 24 human males were moderately depleted of copper, an elevation in plasma cholesterol was not demonstrated. However, an increase in LDL cholesterol level and a reduction in HDL cholesterol level were observed. If the hamsters in the present study were further depleted of copper, a continued shift of cholesterol to LDL may have resulted in similar findings. But at the same time, the further depletion of copper may not have necessarily been advanced by a longer treatment duration. Sherman (1981, as cited in Lei, 1990a) maintained female rats on a copper deficient diet for 18 weeks and still found only small reductions in liver copper stores. It was suggested that because the treatment was initiated one week post-weanling, the rats may have acquired higher copper stores than the weanling rats used in most other successful studies.

Subsequent to the present study, it was discovered that hamster pups open their eyes and are mobile at two weeks of age. At that point, they may begin to consume the mother's stock diet that is known to be much higher in copper than the mother's milk, which is naturally very low in copper. The pup is able to store the copper in the liver, and consumption of the mother's ration for one week may allow for storage of a sizable amount of copper in the liver (The animals in the present study fall in exactly this time category). This could result in the inability to deplete liver copper to a level comparable to that of the previously successful levels in other studies. In illustration of this, a recent study in our lab used two week old hamsters (as compared to the

three week old animals used in Study 1 and 2) and was able to induce a more severe reduction in copper status in roughly the same duration of treatment. Indeed, these animals expressed hypercholesterolemia and hyperlipoproteinemia.

All in all, the results of both Study 1 and Study 2 indicate that the copper status of the animals was not sufficiently reduced in order to induce alterations in cholesterol and lipoprotein metabolism. Moreover, in order to obtain a sufficient depletion in copper by dietary means, hamster pups should be placed on a deficient diet prior to the third week of age.

SUMMARY

In the first experiment, 22 male Golden-Syrian hamsters, three weeks of age were randomly divided into two groups and fed either a copper-adequate (6.40 ppm) or a copper-deficient (0.54 ppm) diet depending on the group. The animals fed a copper-adequate diet gained significantly more weight and consumed more of the diet than those fed the deficient diet. After eight and half weeks, blood was collected from the animals and the plasma lipoproteins were separated and analyzed for protein, cholesterol and triglyceride content. The blood hematocrit and hepatic copper content were determined to be reduced in the copper-deficient treatment, whereas the plasma volume was enlarged. No difference was evident between the groups as to protein, cholesterol and triglyceride concentration or pool size in each of the lipoprotein fractions. The absence of modifications in cholesterol metabolism and lipoprotein composition as a result of the deficient diet seems to have resulted from an insufficient depletion of copper stores in the treatment animals.

In the second experiment, three week old female Golden-Syrian hamsters, 14 in number, were randomly assigned to one of the two dietary treatments as in Study 1: copper-deficient feed (0.54 ppm) or copper-adequate feed (6.40 ppm). The animals were sacrificed after five and a half weeks on the experimental diet, and blood was collected and plasma was separated by lipoprotein classes. Similar to Study 1, the hematocrit and liver copper content decreased, and the plasma volume increased in the deficient animals as compared to the

adequate animals. In contrast to Study 1 and to what would be expected, HDL cholesterol concentration was lower in the deficient group than in the adequate group. When the data were expressed as the amount present in the vascular pool per 100 gram body weight, the HDL protein content remained significantly different between the treatments.

An explanation for these results could be that the small reduction in hematocrit and enlarged plasma volume in the deficient animals may have led to a dilution effect on the HDL fraction, resulting in the appearance of a small decrease in HDL cholesterol concentration. Alterations in lipoprotein component concentrations may follow the change in plasma cholesterol concentration and because this has not been shown to occur much later in moderate deficiency as compared to severe deficiency, the animals in both studies may not have been deficient enough to elicit the metabolic changes. Beginning the treatment one week earlier in the animals stage of life has been suggested.

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