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FRACTION IN RATS

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EFFECT OF COPPER DEFICIENCY ON THE
APOLIPOPROTEIN-E-RICH HIGH DENSITY
LIPOPROTEIN FRACTION IN RATS

by
Susan Corrine Croswell

A Thesis Submitted to the Faculty of the
COMMITTEE ON NUTRITIONAL SCIENCES (GRADUATE)
In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE
In the Graduate College
THE UNIVERSITY OF ARIZONA

1984
STATEMENT BY AUTHOR

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SIGNED: Susan Croswell

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

K. Y. Lei
Associate Professor of Nutrition

Aug 1st, 1984
In memory of my late father

John S. Croswell, Jr.
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ABSTRACT

Twenty-four male weanling rats were randomly divided into two treatment groups, namely a copper-adequate (8 mg Cu/kg diet) or a copper-deficient (0.85 mg Cu/kg diet) group. Feed and distilled, deionized water were provided ad libitum. After 7 weeks, plasma lipid, protein and apolipoprotein concentrations of high density lipoproteins (HDL) and subfractions of HDL were determined. Significant elevations in the protein and cholesterol contents of HDL and the second retained (R\textsubscript{II}) subfraction of HDL were observed in the copper deficient rats. In addition, the apolipoprotein E (apo-E) concentration of HDL and the R\textsubscript{II} subfraction of HDL was significantly increased due to copper deficiency. Copper deficiency also significantly elevated the level of apolipoprotein AI (apo-AI) of the R\textsubscript{II} subfraction. Data obtained in this study suggest that the hypercholesterolemia associated with copper deficiency was due to an impairment in the cholesterol degradation process regardless of an increase in the apo-E-rich HDL fraction.
INTRODUCTION

Although various studies have been performed to elucidate the mechanisms responsible for the hypercholesterolemia observed in copper-deficient rats, the role of copper in cholesterol metabolism is still unknown (Lei 1977, 1978, 1983; Allen and Klevay 1978, 1980). Lei (1977, 1978) suggested that a shift of cholesterol from the liver pool to the plasma pool was responsible for the observed hypercholesterolemia in copper-deficient rats. Allen and Klevay (1978) suggested that a more rapid clearance of cholesterol from the liver to the plasma pool, with this cholesterol being unavailable for excretion as biliary steroids, may be responsible. Subsequently, newly synthesized cholesterol ester was shown to clear the liver faster in copper-deficient rats compared to controls (Shao and Lei 1980). In the copper-deficient rats, Lin and Lei (1981) observed marked increases in the size and half-life of the rapidly exchangeable cholesterol pool, which consisted of tissues that equilibrate rapidly with the serum cholesterol. In addition, there was a prolonged half-life of the free cholesterol and total cholesterol carried by the high density lipoproteins (HDL) of copper-deficient rats (Lei and Lin 1981). Lei (1983) recently observed marked increases in protein and cholesterol contents of HDL and low density lipoproteins (LDL) and in the triglyceride content of LDL due to copper deficiency. Thus, the reduction in the rate of removal of cholesterol
from the circulation and the observed hypercholesterolemia may be due mainly to an impairment in the cholesterol degradation process.

Apolipoproteins are important structural components of lipoproteins, but also function in lipoprotein metabolism by receptor-mediated uptake of lipoproteins (Brown and Goldstein 1976). Alterations of apolipoprotein profiles and/or changes in receptor functions could result in hyperlipidemia. Cholesterol feeding induces increased concentrations of HDL containing apolipoprotein E (apo-E) which are rapidly cleared by the liver through a high-affinity, receptor-mediated process (Mahley 1981). The recent discovery of a hepatic apo-E receptor in mature animals, capable of interacting with HDL containing apo-E, also emphasizes the importance of the apo-E-rich HDL in the pathway of hepatic cholesterol degradation (Hui et al. 1981). Indeed, Lei (1983) observed increased apo-E concentrations of the HDL fraction in copper-deficient rats. Therefore, possible changes in apolipoprotein concentrations and/or distribution may contribute to the observed hypercholesterolemia associated with copper deficiency. This study was designed to determine whether copper status may result in alterations in the distribution of the lipid, protein and apolipoprotein concentrations of HDL and the subfractions of HDL.
Copper was first recognized as an essential dietary component when it was discovered to be essential for hemoglobin synthesis (Hart et al. 1928). The adult human body has been estimated to contain 80 mg of total copper (Cartwright and Wintrobe 1964a). Spray and Widdowson (1951) indicated that new born and very young animals are normally richer in copper per unit of body weight than adults of the same species. The pituitary, thyroid, thymus, prostate gland, ovaries and testes represent tissues of low copper concentration while the liver, brain, kidneys, heart and hair have relatively high copper concentrations (Carlton and Henderson 1963). Yet, Underwood (1977) noted that the distribution of total body copper among the tissues varies markedly in all species.

Copper stored in the liver, the main storage organ of the body for this element, is in highest concentrations in newborns and decreases with age in most species (Underwood 1977). In addition, Gregoriadis et al. (1967) observed changes in copper distribution among subcellular fractions of the liver as the rat matures. At birth over 80% of the total copper is present in the nuclear and mitochondrial fractions, while the supernatant contains about one-half the total copper content of the liver in the adult rat. Copper supplementation of the diets of rats has no comparable effects on liver copper storage.
until copper levels reach 200 ppm. Liver copper levels then increase rapidly possibly due to overloading of the excretory mechanism (Milne and Weswig 1968). Copper retention in the liver and other tissues is influenced by the levels of zinc, cadmium, iron, molybdenum and calcium carbonate in the diet due to their effects on upper intestinal absorption and/or excretion (Underwood 1977). No effect of sex on liver copper concentration has been observed except with the Australian salmon in which the female carries higher levels of copper than the male (Beck 1956).

The copper in blood exists in both erythrocytes and plasma. Shields et al. (1961) found that at least 60% of total red cell copper was bound as erythrocuprein, now known to be the enzyme superoxide dismutase (McCord and Fridovich 1969). About 95% of the copper plasma is present in the form of ceruloplasmin, an α2-globulin which firmly binds 6-8 copper atoms per mole in a non-exchangeable form (Underwood 1977). Ceruloplasmin was identified by Osaki et al. (1966) as an oxidase (ferroxidase) involved in iron utilization and in promoting iron saturation of transferrin in the plasma. The principal transport forms of copper within the plasma are its loosely bound complexes with albumin (Gubler et al. 1957) and, to a lesser extent, with selected amino acids such as histidine, threonine and glutamine (Neumann and Sass-Kortsak 1967).

Pregnancy (Halsted et al. 1968), the administration of oral contraceptives in women (Prasad et al. 1975); stilbesterol in rats; thyroxine in sheep (Underwood 1977); and estradiol in men (Johnson
et al. 1959) had each been shown to elevate plasma copper levels above normal concentrations (0.5 to 1.5 ug/ml; Beck 1961). In addition, plasma copper levels are slightly higher in women than in men (Hambidge and Droegemueller 1974). Trace elements such as zinc, cadmium and iron which depress copper absorption also reduce plasma copper concentrations when ingested at high dietary levels (Underwood 1977). The effect of both molybdenum and sulfate depends on the status of the animal with respect to these elements and copper (Gray and Daniel 1964). Milne and Weswig (1968) observed no increase in plasma copper in rats when the copper content of the diet was raised from 10 to 50 ppm, whereas a copper intake of 100 ppm doubled plasma concentrations. Severe hypercupremia in pigs develops with highly toxic intakes, such as 750 ppm copper, which can be prevented by the administration of 500 ppm zinc (Suttle and Mills 1966).

Van Campen and Mitchell (1965) suggested copper was absorbed from the stomach and all portions of the small intestine in the rat. Thompsett (1940) indicated that in man copper was absorbed particularly in the upper small intestine, the duodenum. In sheep most copper is absorbed in the large intestine (Grace 1975). Yet in almost all species, dietary copper is poorly absorbed. Major factors affecting the absorption of copper include: (1) the pH of intestinal contents; (2) the dietary level of organic chelating agents and other minerals; (3) the chemical form of the copper ingested; and (4) the age of the animal (Underwood 1977). An increase in intestinal pH (Thompsett 1940); the formation of insoluble oxides (Lassiter and Bell 1960) and stable
complexes with phytates (Davis et al. 1962); an elevated intake of ascorbic acid (Van Campen and Gross 1968); cadmium, zinc, iron and molybdenum (Underwood 1977); and an increase in the age of the animal (Suttle 1973) all markedly reduce copper absorption. The mechanisms regulating copper absorption are not described, although it seems that metal-binding factors are involved and that the inhibition of copper absorption by various metals previously described, may result from competition for protein metal-binding sites (Underwood 1977). Starcher (1969) identified a single metal-binding protein in chick duodenum which would bind copper, as well as cadmium and zinc. Subsequently, Evans and Hahn (1974) found orally administered copper to be associated with a variety of metal-binding ligands and macromolecules in the intestine of the rat. In the intestinal lumen copper was complexed with a protein similar to metallothionein, yet it is not clear whether this protein is actually involved in copper transport from the intestine to the blood.

Studies of genetically defective copper metabolism in humans and animals (Danks et al. 1973) indicated that the regulation of copper absorption involves two distinct mechanisms, copper transport from the intestinal lumen to the mucosal cells and from the mucosal cells to the plasma. Copper entering the plasma from the intestine is loosely bound to serum albumin and specific amino acids, and a direct reacting copper pool is formed from which copper is widely distributed to various tissues (Bush et al. 1956).
The liver is the major organ of copper metabolism. Copper is incorporated into components of liver parenchymal cells in proportions which vary with the age and copper status of the animal (Porter et al. 1961). Copper may either be stored in the liver or released for incorporation into erythrocuprein (Shields et al. 1961), ceruloplasmin (Wintrobe et al. 1953) and other copper-containing enzymes (cuproenzymes). The liver also provides the major excretory pathway of copper via the bile (Underwood 1977).

Anemia is a common expression of severe and prolonged copper deficiency in all species. In most copper-deficient animals the anemia is hypochromic and microcytic (Underwood 1977). Copper deficiency appears to impair erythrocyte maturation (Lahey et al. 1952) and integrity without involvement of the heme biosynthetic enzymes (Lee et al. 1968). The transport of iron from tissue to plasma may be impaired due to the reduction in ceruloplasmin activity necessary for the oxidation of ferrous to ferric iron in the formation of Fe$^{3+}$ transferrin (Osaki et al. 1966).

Neonatal ataxia has been observed in copper-deficient lambs, goats, pigs and rats (Underwood 1977). Fell et al. (1965) suggested that this condition was due to a deficiency of cytochrome oxidase, the copper-containing terminal respiratory enzyme, in the motor neurons. Gallagher et al. (1956) concluded from studies of copper-deficient rats that the loss of cytochrome oxidase activity is due to a lack of synthesis of its prosthetic group, heme. Demyelination associated with neonatal ataxia is attributed to depressed cytochrome oxidase activity.
which may lead to inhibition of aerobic metabolism and phospholipid synthesis (Gallagher and Reeve 1971).

A nervous disorder of lambs, "swayback" disease, characterized by uncoordinated movement has been recognized in various parts of the world (Underwood 1977). Alloway (1973) suggested that molybdenum-induced hypocuprosis may be a contributory factor in the incidence of the disease.

Changes in the growth and appearance of hair, fur and wool (achromotrichia) are observed in copper-deficient rats, rabbits, guinea pigs, dogs, cattle and sheep. O'Dell (1976) suggested the lack of pigmentation is due to a reduction in the activity of the copper-containing polyphenyl oxidases which catalyze the conversion of tyrosine to melanin. Impaired keratinization, characterized by the appearance of abnormally straight, "stringy" hair, seems to be due to reduced incorporation of disulfide groups in keratin synthesis (Underwood 1977).

Copper deficiency results in reproductive failure due to fetal death and resorption in rats and guinea pigs (Howell and Hall 1969). Low fertility in cattle grazing copper-deficient pastures, associated with delayed or depressed estrus has been observed in several areas (Allcroft and Parker 1949).

The first evidence of cardiovascular disorders in copper deficiency emerged from studies of a disease in cattle known as "falling disease" (Bennetts and Hall 1939). More recently, Gubler et al. (1957) reported sudden cardiac failure associated with cardiac hypertrophy in
copper-deficient pigs and rats. Studies of copper-deficient pigs by Coulson and Carnes (1963) demonstrated that extensive internal hemorrhage resulting in sudden death was due to an impairment in arterial vessel structure. O'Dell et al. (1961) observed a derangement of the elastic tissue in the aortas of copper-deficient chicks while other investigators (Carlton and Henderson 1962; Simpson and Harms 1964) subsequently observed aortic rupture with degeneration of the elastic membranes in such animals. These and other findings have combined to elucidate the roles of copper in elastin (collagen) biosynthesis (Underwood 1977). Reduced levels of elastin and collagen were observed in the aortas of copper-deficient animals (Weisman et al. 1963; Starcher et al. 1964). Collagen and elastin from such animals contained elevated levels of lysine and decreased levels of desmosine apparently due to a reduction of lysyl oxidase activity (Miller et al. 1965; O'Dell et al. 1966). Copper-containing lysyl oxidase catalyzes the condensation of lysine residues (Chou et al. 1969) to form desmosine, the key cross-linkage group in elastin and collagen (Partridge et al. 1964). Thus, in copper deficiency fewer cross-linkages are present in the connecting tissue resulting in less elasticity of the aorta (Hill et al. 1968).

Fatty acid metabolism is impaired in copper deficiency. Wahle and Davies (1975) demonstrated decreased monounsaturated:saturated ratios for C16 and C18 fatty acids from subcutaneous adipose tissue and decreased desaturase activity in liver microsomes of young copper-deficient rats.
Dietary copper deficiency is rare in the adult population of the United States (Cartwright and Wintrobe 1964b). Yet copper deficiency has been reported in premature infants fed exclusively on modified cows' milk (Al-Rashid and Spangler 1971) and in infants during prolonged parental alimentation (Karpel and Peden 1972). Recent surveys of a variety of American diets indicated copper intakes of less than 1 mg/day which is considerable below that of the recommended 2-3 mg/day for adults (Klevay 1975).

**Lipoprotein Metabolism**

Only 7% of the body's total cholesterol circulates in plasma (Goldstein and Brown 1982). Compelling evidence implicates high plasma cholesterol levels as a major cause of atherosclerosis (Goldstein and Brown 1977). Human hypercholesterolemia is caused by genetic or acquired abnormalities in the synthesis or degradation of plasma lipoproteins that transfer endogenous cholesterol between body tissues. In laboratory animals, hypercholesterolemia is produced exogenously when normal mechanisms of lipoprotein clearance are saturated by large amounts of dietary cholesterol. Since exogenous and endogenous cholesterol are carried by different plasma lipoproteins in separate transport pathways, the analogy between the cholesterol-fed animal model and human atherosclerosis may be imperfect (Goldstein et al. 1983).

The major transport lipoproteins of endogenous cholesterol in human plasma are low density lipoproteins (LDL) (Goldstein et al. 1983). These lipoproteins leave the liver in precursor forms known as
very-low density lipoproteins (VLDL) (Havel et al. 1980). The core of VLDL contains mostly triglycerides with small amounts of cholesteryl esters and its surface holds three proteins (apolipoproteins): apolipoprotein B-100 (apo-B-100), apolipoprotein E (apo-E) and apolipoprotein C (apo-C). In the capillaries of adipose tissue and muscle, the VLDL triglycerides are hydrolyzed by an endothelial enzyme, lipoprotein lipase, which releases apo-C and produces triglyceride-depleted remnant particles that are thereby converted to intermediate density lipoproteins (IDL) fairly enriched in cholesteryl esters. The IDL dissociate from the endothelium and return to the circulation. While some of these particles are rapidly cleared from the plasma by hepatic uptake, others remain in the circulation where most of their residual triglycerides are removed, leaving cores of nearly pure cholesteryl ester. Apo-E leaves the particles and apo-B-100 remains during this final conversion to low density lipoproteins (LDL) (Havel et al. 1980). Two-thirds of the LDL are metabolized after binding to specific LDL (apo-B,E) receptors located on the surface of the liver and extrahepatic cells (Goldstein and Brown 1983) which recognize the apo-B-100. Binding leads to cellular uptake and lysosomal degradation of the LDL receptor by receptor-mediated endocytosis. The remaining one-third of the LDL not taken up by the receptor are metabolized by alternative receptor-independent mechanisms, some of which are expressed in macrophages and other scavenger cells of the reticuloendothelial system (Goldstein and Brown 1983).
In animals resistant to atherosclerosis, such as the rat, the majority of endogenous cholesterol is transported by high density lipoproteins (HDL) (Mahley 1978). HDL have been hypothesized to play an essential role in returning peripheral cell cholesterol to the liver by a reverse cholesterol transport process (Sloop et al. 1983). It appears that the liver secretes a nascent (discoidal) HDL containing mostly phospholipids and apolipoproteins E and A. In the blood or periphery, the disc picks up free cholesterol from cell membranes and from excess surface of triglyceride-rich particles in their conversion to remnant particles. Once in the plasma, the enzyme lecithin:cholesterol acyltransferase generates cholesteryl esters from these lipids, producing a spherical HDL particle with a cholesteryl ester-rich core (Glomset 1968). The cholesteryl esters can also be transferred rapidly to remnant particles during lipolysis of the triglycerides. HDL and remnants may then be rapidly recognized and removed by hepatic apo-E receptors. In humans, lipoprotein remnants are mostly converted to IDL then LDL (Siggurdsson et al. 1975), whereas in rats most are catabolized by the liver (Havel 1975). HDL may unload their cholesteryl ester contents at the hepatocyte membrane and recycle many times to the periphery prior to degradation (Drevon et al. 1977).

The transport pathway for exogenous cholesterol differs from the endogenous pathway. Dietary cholesterol is incorporated into chylomicrons, whose apolipoprotein B-48 (apo-B-48) is of lower molecular weight than the apoprotein B-100 of VLDL and LDL (Kane et al. 1980). After their triglycerides are hydrolyzed by endothelial lipoprotein
lipase, the cholesteryl ester-rich chylomicron remnants are rapidly taken up by the liver through receptor-mediated endocytosis. Neither dietary particle is converted to LDL.

The primary control mechanisms for the homeostasis of lipoproteins in the plasma—and hence for homeostasis of plasma cholesterol and triacylglycerols—are the lipoprotein receptors. Brown and Goldstein were pioneers in establishing the existence of lipoprotein receptors, which bind LDL, on the surface of fibroblasts (Goldstein and Brown 1977). The LDL bind to the cell surface receptors, apo-B,E receptors, and are localized in areas of coated pits. These coated pits invaginate, forming coated vesicles which enter the cell and fuse with lysosomes. The LDL are hydrolyzed, liberating amino acids from apo-B and free cholesterol from the cholesteryl esters. It appears that the free cholesterol migrates into the cytoplasm, where it elicits three intracellular regulatory responses that maintain cholesterol homeostasis: (1) the suppression of hydroxymethyl glutaryl-CoA reductase, the rate-limiting step in cholesterol biosynthesis, (2) the activation of acyl CoA-cholesterol acyl transferase (ACAT) which esterifies free cholesterol for storage inside the cell, and (3) the down-regulation of the expression of LDL receptors. The number of receptors controls the entry of LDL cholesterol, thereby providing both the means for acquiring the cholesterol for various cellular functions (synthesis of membranes, steroid hormones and bile acids) and a mechanism to protect against excessive accumulation of cholesterol or cholesteryl esters (Mahley and Innerarity 1983).
Familial hypercholesterolemia (FH) is one of the most common genetic diseases affecting man (Goldstein and Brown 1983). In vitro studies of cultured human fibroblasts have established that the primary defect in FH lies in the gene for the LDL receptor (Tolleshaug et al. 1983). Combined biochemical and genetic studies have led to the identification of at least seven allelic mutations, the most frequent of which leads to an absence of any functional receptor protein. The receptor-mediated endocytosis and lysosomal degradation of plasma LDL appears to be absent or markedly reduced and the plasma LDL level rises, thereby leading to an accumulation of endogenous cholesterol and, ultimately to atherosclerosis. The LDL levels of heterozygotes and homozygotes with FH are 2 to 3-fold and 6 to 8-fold above normal, respectively (Goldstein and Brown 1982). A majority of patients with high LDL levels have functional LDL-receptor genes and appear to have a form of endogenous hypercholesterolemia produced by exogenous factors, including dietary cholesterol. Experiments in rabbits (Kovanen et al. 1981a) and in dogs (Hui et al. 1981) have shown that a high-cholesterol diet causes cholesterol to accumulate in the liver, thus suppressing hepatic production of LDL (apo-E) receptors. Cholesterol feeding also induces a reduction in the level of regular HDL (HDL without apo-E) and an increase in the level of HDL-C (HDL with apo-E and cholesterol ester-rich) in many species (Mahley 1978), thereby enhancing cholesterol transport to the liver. Hepatic LDL receptors can also be suppressed in rabbits fed a cholesterol-free diet composed of carbohydrate and casein and this suppression contributes to the high LDL.
levels observed in the absence of dietary cholesterol (Chao et al. 1982). In dog, swine and man, the expression of hepatic LDL (apo-B,E) receptors has been shown to decrease with age (Mahley et al. 1981) suggesting an explanation for the age-related increase in plasma LDL levels that occurs in humans. Thus, a variety of nongenetic factors may contribute to the elevated LDL levels by suppressing the synthesis of hepatic LDL receptors.

Various therapeutic, receptor-stimulating methods have been used to deplete the liver of cholesterol, thereby inducing an increase in the expression of (apo-B,E) LDL receptors (Goldstein and Brown 1982). Cholestyramine, a bile acid-binding resin, prevents the re-utilization of bile acids through enterohepatic circulation, thus forcing the liver to convert more cholesterol into bile acids (Dietschy and Wilson 1970). The drug compactin is an extremely potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-controlling enzyme in cholesterol synthesis (Brown et al. 1978). Used in combination, these drugs have been successful in limited clinical studies using dogs and may prove to be a powerful therapy for hypercholesterolemia (Kovanen et al. 1981b).

Recently, a unique receptor, specific for the recognition of the E apoprotein, was reported in adult canine liver (Hui et al. 1981). As previously mentioned, adult animals lack apo-B,E receptors, but possess the apo-E receptor capable of interacting with HDL containing apo-E and chylomicron remnants. However, immature animals possess both apo-B,E and apo-E receptors (Mahley et al. 1981). The expression of
the apo-E receptor remains relatively constant for all ages of animals studied and is not strictly regulated by metabolic perturbations (i.e. cholesterol feeding, cholestyramine) (Mahley et al. 1981). These observations demonstrate the importance of the apo-E receptor in lipoprotein metabolism and cholesterol homeostasis, especially in the aging animal.

The apo-B,E receptor interacts not only with LDL containing apo-B, but also with HDL containing apo-E which suggests that the interaction of the lipoproteins with the LDL receptor depends on a mechanism involving apo-B or apo-E (Innerarity and Mahley 1978). The importance of these protein moieties (apo-B and apo-E) in mediating the interaction of the lipoproteins with the receptors was established by studies in which specific amino acid residues of these apoproteins were chemically modified. The selective modification of a limited number of arginine or lysine residues of LDL and HDL containing apo-E resulted in the loss of receptor binding activity (Mahley et al. 1980) demonstrating the importance of these particular amino acid residues in cellular recognition and uptake of lipoproteins. Furthermore, an understanding of the way in which apo-E interacts with the apo-B,E receptor has recently progressed due to the elucidation of the structure and genetics of apo-E. Human apo-E exists in three major forms, E-2, E-3 and E-4 isoforms, as determined by isoelectric focusing on acrylamide gels (Weisgraber et al. 1982). Patients with Type III hyperlipoproteinemia, a genetic disease associated with defective plasma lipoprotein clearance, possess the E-2 isoform (Fredrickson et al. 1978).
The apo-E-2, a mutant isoform, demonstrates impaired apo-B,E receptor binding activity (Weisgraber et al. 1982) which contributes to the abnormal lipoprotein clearance and the development of hyperlipidemia. Detailed amino acid and sequence analyses of apo-E revealed that the major isoforms of apo-E differ from one another by single amino acid substitutions of cysteine or arginine and account for the relative charge differences observed in isoelectric focusing (Rall et al. 1982). These studies of the mutant forms of apo-E have been useful in determining the region of the molecule responsible for receptor binding. However, as with endogenous hypercholesterolemia other non-genetic factors may influence the effects of the mutant E-2 protein on lipoprotein metabolism.

**Copper Deficiency and Cholesterol Metabolism**

Recently, the biochemical role of copper in the regulation of cholesterol metabolism has been a focus of nutritional investigation. Murthy et al. (1972) suggested an important relationship between dietary zinc and copper on lipid metabolism in rats. However, the hypercholesterolemic rats were copper-deficient. In addition, Klevay (1973) induced hypercholesterolemia in rats by an increase in the ratio of zinc to copper ingested. He recently hypothesized that a high ratio of dietary zinc to copper is associated with hypercholesterolemia and further postulated that coronary heart disease is predominantly a disease of imbalance of zinc and copper metabolism (Klevay 1975). In two large factorial animal experiments, using various levels of dietary
zinc and copper, Petering et al. (1977) and Murthy and Petering (1976) demonstrated in rats that levels of serum cholesterol, triglyceride and phospholipids were inversely related to dietary copper as well as serum copper. There was no significant relationship of serum or dietary zinc and serum cholesterol, however, at low serum copper levels due to low dietary intake of copper, an excessive intake of zinc appeared to raise the serum cholesterol levels due to the zinc-copper interrelationship. Therefore, Petering and Murthy (1976) suggested that dietary copper played a greater role in the metabolism of cholesterol, much more so than the zinc to copper ratio.

Subsequently, various investigators have attempted to elucidate the mechanisms responsible for the hypercholesterolemia observed in copper-deficient rats. An increase in hepatic cholesterol synthesis appeared unlikely since the in vitro rate of cholesterogenesis from acetate-1-14C in liver slices was not enhanced by copper deficiency (Lei 1977). Furthermore, no alteration in cholesterol degradation in vivo (Lei 1978) or biliary steroid excretion (Allen and Klevay 1978) was observed in copper-deficient rats. Lei (1977, 1978) suggested that a shift of cholesterol from the liver pool to the plasma pool appeared to be responsible for the hypercholesterolemia observed in cholesterol-deficient rats. Allen and Klevay (1978) also suggested that a more rapid clearance of cholesterol from the liver to the plasma pool, with this cholesterol being unavailable for excretion as biliary steroids may be responsible for the hypercholesterolemia in copper-deficient rats. Shao and Lei (1980) demonstrated that cholesterol ester, newly
synthesized from mevalonate-2-\(^{14}\)C, cleared the liver faster in copper-deficient rats compared to controls. Using a kinetic-two-pool analysis of serum cholesterol, Lin and Lei (1981) further observed marked increases in the size and half-life of the rapidly exchangeable cholesterol pool in copper-deficient rats as compared to controls. There was also a prolonged half-life of the free cholesterol and total cholesterol carried by the high density lipoproteins (HDL) of copper-deficient rats (Lei and Lin 1981). Lei (1983) recently observed significant elevations in protein and cholesterol contents of HDL and low density lipoproteins (LDL) and in the triglyceride content of LDL due to copper deficiency. In addition, the apolipoprotein E concentration of HDL was markedly increased in the copper-deficient rats.
MATERIALS AND METHODS

Experimental Design

Twenty-four male weanling Sprague-Dawley rats weighing between 40 and 54 gm were equally and randomly divided into two treatment groups (copper-adequate and deficient) with diets containing 8 mg Cu/kg and 0.85 mg Cu/kg, respectively. The basal diet (copper-deficient) was similar to that recommended by the American Institute of Nutrition (1977, 1980) except that the mineral mix was not supplemented with copper (Table 1). The basal diet contained 0.85 mg of Cu per kg of diet as measured by flameless atomic absorption spectrophotometry using a NSA Hitachi model 180-70 (Mountain View, CA). The copper-adequate diet was prepared by adding copper carbonate to the basal diet to provide a final concentration of 8 mg of Cu per kg of diet. The respective diets and distilled-demineralized water (distilled water passed through a mixed-bed resin, Barnstead Co., Sybron Corp., Boston, MA) were provided ad libitum.

Rats were housed individually in suspended stainless-steel wire cages in a laboratory maintained at 22°C with 12 hours each of light and darkness. Feed consumption was measured weekly and body weights were recorded throughout the experiment.

After seven weeks of treatment the rats were fasted overnight, anesthetized with ether and killed. During the treatment period, one of the rats from the copper-deficient group died and some blood samples
Table 1. AIN-76\textsuperscript{TM} Purified Diet (for rats and mice).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percent</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>200</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>Glucose Monohydrate</td>
<td>65.0</td>
<td>650</td>
</tr>
<tr>
<td>Fiber</td>
<td>5.0</td>
<td>50</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>5.0</td>
<td>50</td>
</tr>
<tr>
<td>AIN Mineral Mix\textsuperscript{1}</td>
<td>3.5</td>
<td>35</td>
</tr>
<tr>
<td>AIN Vitamin Mix\textsuperscript{2}</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{1}See Appendix A, Table 1 for composition

\textsuperscript{2}See Appendix A, Table 2 for composition

from the copper-adequate group were lost during processing. Blood was obtained by cardiac puncture and plasma was rapidly isolated and processed for lipoprotein separation. The heart and liver were quickly excised and weighed. One gram liver samples were obtained from each liver, dried at 80\textdegree C for 3 days, digested in 3 ml of nitric acid at 95\textdegree C for 3 hours and made up to a final volume of 5 ml. Liver copper concentrations were determined by flameless atomic absorption spectrophotometry.
Biochemical Procedures

Plasma Lipoprotein Separation

Plasma lipoproteins were separated and purified by the method of Rudel et al. (1974) using ultracentrifugation and agarose-column chromatography. Blood was mixed with EDTA (final concentration 2.6 mM) and plasma was removed immediately by centrifugation at 1000 x g at 15°C for 15 minutes. Equal volumes of plasma from 3 animals were pooled to obtain a total of 10 ml. The plasma density was raised to d 1.225 by adding solid KBr (0.3517 gm of KBr/ml of plasma). Ten milliliters of plasma were placed in ultracentrifuge tubes and overlayered with a d 1.225 buffered solution (Scanu and Granada 1966). Tubes were centrifuged in a type 30 rotor for 24 hours at 15°C at 105,000 x g in a model L ultracentrifuge (Beckman, Fullerton, CA). The top 2 ml containing the lipoprotein concentrate was quantitatively removed. The lipoprotein concentrate was promptly applied to an agarose chromatography column.

Columns (2.6 cm internal diameter with a bed height of 85 cm) were packed with sepharose CL-4B (Pharmacia Inc., Piscataway, NJ) and housed in a cold room maintained at 6°C. Less than 75 mg of lipoprotein cholesterol in a 2-4 ml volume were applied to each column and eluted at a rate of 21 ml/hr with 0.15 M NaCl, 0.01% EDTA, 0.02% NaAzide, pH 7.0. Fractions of the eluate were collected by an LKB model 2111 fraction collector (LKB instruments Inc., Rockville, MD) and the protein component was monitored at 280 nm. Three individual peaks of lipoproteins were eluted (peak 1, very-low density, VLDL; peak 2,
low density, LDL; peak 3, high density, HDL). Fractions from the HDL peak were combined and concentrated to 2 ml by ultrafiltration using YM 30 filters in Amicon stirred cells (Amicon Corp., Danvers, MA). One ml of the concentrated HDL was used for affinity chromatography which is equal to the amount of HDL from 5 ml of original rat plasma. The other half of the concentrate was stored for protein, cholesterol, triglyceride and apolipoprotein determinations.

Subfractionation of Plasma HDL

Plasma HDL were subfractionated according to the method of Weisgraber and Mahley (1980) using heparin-Sepharose affinity chromatography. A column (1.0 x 30 cm) was prepared by using heparin-Sepharose CL-6B (Pharmacia, Inc., Piscataway, NJ) and maintained at 6°C. The heparin-Sepharose column was equilibrated with NaCl-Tris buffer (0.05 M NaCl, 0.005 M Tris, pH 7.4) containing 0.025 M MnCl₂. Approximately 5 mg of protein in 1 ml of plasma HDL concentrate were applied to the column and allowed to stand overnight. The column was eluted by an increasing step-gradient system at a rate of 24 ml/hr. Fractions of the eluate were collected by a LKB model 2111 fraction collector and the protein component was monitored at 280 nm. The non-retained lipoproteins (NR peak, fractions 4-8) were eluted with the manganese-containing buffer, 0.05 M NaCl-Tris buffer, until fraction 11 had been collected. At this point the NaCl concentration of the elution buffer was increased to 0.07 M and the manganese was deleted. This resulted in the elution of the first fraction of retained HDL.
(R_1 peak, fractions 17-21). An increase in the NaCl concentration to 0.29 M at fraction 27 eluted the second fraction of retained HDL (R_{II} peak, fractions 32-36). A final increase in the NaCl concentration of the eluent to 0.6 M was made at fraction 37. The subfractions of HDL (NR, R_1 and R_{II}) were dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.0 and concentrated to 1 ml by the Amicon Ultrafiltration System using YM 5 filters.

Apolipoprotein Composition of HDL and its Subfractions

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the apolipoprotein composition of HDL and its subfractions. A gradient slab gel of 7.5 to 20% acrylamide with a stacking gel of 4.5% was used according to the method of Laemmli (1970) to separate the apolipoproteins. The protein contents of HDL and its subfractions were determined prior to application. Approximately 25 ug of HDL, 15 ug of the NR fraction, 35 ug of each of the retained fractions and 30 ug of molecular weight standard were applied and run on a Hoefer model SE 400 vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA) at 25 mA. The 5 ug of SDS-PAGE low weight molecular standard (#25678, MW range 10,000-100,000, Biorad Laboratories, Richmond, CA) contained 5 ug each of phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. Protein bands were stained with 0.2% coomassie blue. Apolipoprotein concentrations were quantified by scanning the stained gels at 660 nm with a Zeiss model PM6 spektralphotometer and then measuring the areas under
the peaks. The concentrations were calculated on the basis of total protein applied to each gel. The quantity of individual apolipoprotein should be directly proportional to the dye bound to each apolipoprotein. This was validated by running a series of gels with known and increasing amounts of apolipoproteins (Dory and Roheim 1981).

Procedure for Plasma Protein Determination

The protein concentration of HDL and its subfractions were measured colorimetrically using the methods of Lowry et al. (1951). Graded levels of protein standards, 0, 25, 50, 100, 150 and 200 mg were used to construct a standard curve. Bovine albumin (A4378, crystallized and lyophilized, Sigma Chemical Co., St. Louis, MO) was used as the protein standard. Plasma protein in solution reacted with copper in alkaline solution. The products formed were used to quantitatively reduce a phosphomolybdic-phosphotungstic reagent. The blue color complex was read at 750 nm against a blank using a Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

Procedure for Plasma Cholesterol Determination

Plasma cholesterol levels were quantitatively determined by the method of Bucolo and David (1973) using an enzymatic kit (Boehringer Mannheim, Indianapolis, IN). Certified standards of cholesterol, 50, 100, 125, 150 and 200 mg/dl (Boehringer Mannheim) were used to construct a standard curve. Cholesteryl esters in the samples were split quantitatively yielding free cholesterol, which, in turn was oxidized
to cholest-4-en-3-one producing equivalent hydrogen peroxide.

Methanol, in the presence of hydrogen peroxide and catalase, was converted to formaldehyde, which formed a yellow product, 3,5-diacetyl-1,4-dihydrolutidine, via the Hantzsch (Nash 1953) reaction. The yellow color was read at 410 nm against a blank using a Beckman model 25 spectrophotometer.

Procedure for Plasma Triglyceride Determination

Plasma triglyceride values were measured colorimetrically by the method of Roeschlau et al. (1974) using an enzymatic kit from Boehringer Mannheim (Indianapolis, IN). A certified standard (Boehringer Mannheim) was used. Plasma triglycerides were hydrolyzed to fatty acids and free glycerol using the lipase/esterase solution. The liberated glycerol was phosphorylated and oxidized generating NADH quantitatively. The NADH then reacted with (MTT) 3-(4,5-Dimethyl Thiazoly1-2)-2,5 Diphenyl Tetrazolium Bromide yielding the reduced form (MTT•H). The reduced MTT was measured at 560 nm against a blank using a Beckman model 25 spectrophotometer.

Statistical Analysis

Differences between treatment and control values for all data were calculated. These values were separated into copper-adequate and deficient groups. A student's "t" test was used to statistically analyze the data. A pooled variance estimate was used when the variances were homogeneous and a separate variance estimate was used when the variances were heterogeneous.
RESULTS AND DISCUSSION

Cardiac hypertrophy (Hill 1969; Goodman et al. 1970), depressed growth (Lei 1977; Shao and Lei 1980) and depleted hepatic copper stores (Lei 1977; Tsai and Lei 1979) are well-established indicators of copper deficiency. These abnormalities were also observed in this study. Significant increases in heart weight and heart-to-body weight ratio (Table 2), with reductions in body weight gain and liver copper content (Table 2), were observed in rats fed the copper-deficient diet. Thus, the rats fed the copper-deficient diet were indeed copper-deficient.

Typical elution profiles of lipoproteins separated by agarose-column chromatography are shown in Figure 1. Lipoproteins from 10 ml of plasma derived from copper-adequate or deficient rats were applied to the column. The lipoprotein profiles derived from copper-deficient rats appeared to have elevated lipoprotein levels. The magnitude of the elevated lipoprotein profile was most prominent for VLDL and HDL fractions.

The separation of HDL by heparin-Sepharose column chromatography using the stepwise elution system resulted in a highly reproducible profile of HDL subfractions. A non-retained fraction, NR, was eluted with 50 mM NaCl, 5 mM Tris-Cl and 25 mM MnCl₂. Two retained fractions R₁ and R₁I, were eluted with buffers containing 70 and 290 mM NaCl, respectively, 5 mM Tris-Cl and no MnCl₂. In Figure 2, typical elution profiles of HDL subfractions are depicted. These profiles were
Table 2. Influence of Dietary Copper on Body, Heart and Liver Weights and on Liver Copper Concentrations

<table>
<thead>
<tr>
<th>Wt. or conc.</th>
<th>Copper adequate (n=9)</th>
<th>Copper deficient (n=11)</th>
<th>P-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Wt., g</td>
<td>48.6 ± 1.6</td>
<td>50.4 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Feed consumption per wk (7 wks)</td>
<td>113.5 ± 2.73</td>
<td>104.9 ± 2.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Final Body wt, g/rat</td>
<td>329.9 ± 11.2</td>
<td>243.5 ± 7.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body Wt gain, g/rat, 7 wk</td>
<td>281.3 ± 10.7</td>
<td>193.1 ± 7.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart wet wt, g</td>
<td>1.13 ± 0.05</td>
<td>1.67 ± 0.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart/body wt, %</td>
<td>0.34 ± 0.1</td>
<td>0.68 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver wet wt, g</td>
<td>8.94 ± 0.3</td>
<td>9.08 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Liver/body wt, %</td>
<td>2.71 ± 0.4</td>
<td>3.75 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver copper, mg/whole liver</td>
<td>32.9 ± 2.2</td>
<td>13.4 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^1\)Means ± SEM

\(^2\)P-values derived from simple T tests. A pooled variance estimate was used when the variances were homogeneous and a separate variance was used when variances were heterogeneous.

n=no. of observations

NS=non significant
Figure 1. Agarose Gel Chromatography of Rat Plasma Lipoproteins Fractioned on a Sepharose CL-4B Column (2.6 x 85 cm). Plasma was pooled from three animals to obtain a total of 10 ml for each treatment group and then floated by centrifugation (d>1.225). The resultant lipoproteins were applied to the column, eluted with 0.15 M NaCl, pH 7.0, containing 0.02% NaAzide, 0.01% EDTA and run at a rate of 21 m/hr. Each fraction contained 8.4 ml.
Figure 2. Heparin-Sepharose Affinity Chromatography of HDL from Copper-Adequate and Deficient Rats. Approximately 5 mg of HDL protein in 5 mM Tris-Cl, pH 7.4, containing 50 mM NaCl and 25 mM MnCl₂ were applied to a column (1.0 x 30 cm). HDL were eluted by an increasing NaCl step gradient at a rate of 24 ml/hr. Each fraction contained 4.0 ml.
derived from the amount of HDL obtained from the same volume of plasma (5 ml) for both treatment groups. Most of the HDL applied was recovered in the retained fractions, \( R_I \) and \( R_{II} \). Both \( R_I \) and \( R_{II} \) fractions appeared to be elevated in the copper-deficient group.

However, significant increases in the mean concentrations of protein and cholesterol were observed for HDL and its \( R_{II} \) subfraction, only, due to copper deficiency (Table 3). The increased protein and cholesterol levels of the \( R_{II} \) fraction appeared to contribute to the overall increases in HDL concentrations. Similar increases in the protein and cholesterol contents of HDL were observed in previous studies of copper-deficient rats (Lei and Lin 1981; Lei 1983; Allen and Klevay 1980). In addition, copper deficiency did not significantly alter triglyceride levels of HDL or any of its subfractions.

In the control group, the \( NR, R_I \) and \( R_{II} \) subfractions were found to carry 7.2%, 74.7% and 18.1% of total HDL protein, respectively. In copper deficiency, the percentage was reduced to 62.5% for the \( R_I \) fraction and increased to 11.0% and 26.5% for the \( NR \) and \( R_{II} \) fractions, respectively. However, investigators (Marcel et al. 1981) studying normal human HDL and its subfractions found that the \( NR \) fraction contained the most HDL protein, 78%, while the two retained fractions, \( R_I \) and \( R_{II} \) contained only 18% and 2.5% protein, respectively. In determining cholesterol content of the subfractions, they observed the highest concentration in the \( R_{II} \) fraction, then the \( R_I \) fraction, and lowest concentration of cholesterol in the \( NR \) fraction. This is similar to the distribution of cholesterol among HDL.
### Table 3. Distribution of Protein, Cholesterol and Triglyceride in HDL and Subfractions of Control and Copper-deficient Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HDL Subfraction</th>
<th>Copper Adequate (n=3)</th>
<th>Copper Deficient (n=4)</th>
<th>P-value&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein, mg/100 ml</strong></td>
<td>HDL</td>
<td>83.5 ± 11.1</td>
<td>142.2 ± 5.6</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>6.0 ± 1.7</td>
<td>15.6 ± 7.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;I&lt;/sub&gt;</td>
<td>62.4 ± 11.8</td>
<td>88.9 ± 9.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>15.1 ± 1.0</td>
<td>37.7 ± 1.7</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Cholesterol, mg/100 ml</strong></td>
<td>HDL</td>
<td>48.0 ± 3.8</td>
<td>71.0 ± 3.6</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>2.3 ± 1.4</td>
<td>3.1 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;I&lt;/sub&gt;</td>
<td>27.7 ± 5.2</td>
<td>30.0 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>18.0 ± 1.6</td>
<td>37.9 ± 4.9</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Triglyceride, mg/100 ml</strong></td>
<td>HDL</td>
<td>15.8 ± 4.0</td>
<td>22.3 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>1.6 ± 0.5</td>
<td>4.0 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;I&lt;/sub&gt;</td>
<td>6.5 ± 1.8</td>
<td>10.7 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>R&lt;sub&gt;II&lt;/sub&gt;</td>
<td>7.7 ± 3.4</td>
<td>7.6 ± 3.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean ± standard error of the mean

<sup>2</sup>P-values derived from simple T test

n=number of observations

NS=non significant

NR=non retained peak

R<sub>I</sub>=first retain peak

R<sub>II</sub>=second retained peak
subfractions of the copper-deficient rats, which carried 53.4%, 42.3% and 4.4% of the total HDL cholesterol in the RII, R1 and NR fractions, respectively. In the control group, most of the cholesterol was carried in the R1 fraction, 57.7%, with 37.5% carried by the RII fraction and 4.8% carried by the NR fraction. Thus, there appears to be a difference between human and rat HDL subfraction profiles and their protein and cholesterol content. The NR subfraction of human HDL contained apo-AI, AII, E and C (Marcel et al. 1981) whereas the NR subfraction of both copper-adequate and deficient rats contained only albumin (Table 4). This may contribute to the observed differences in protein content of HDL subfractions.

SDS acrylamide gradient gels of HDL from copper-adequate and deficient rats were densitometrically scanned and a typical profile is shown in Figure 3. Equal amounts of protein were applied to each gel. From the scanning profiles it appears that apolipoprotein levels are elevated due to copper deficiency yet, only apo-E concentrations were significantly increased (Table 4). Lei (1983) previously observed a similar increase in the apo-E concentration of HDL in copper-deficient rats. It is also interesting to note that Van Lenten and Roheim (1982) found elevated concentrations of apo-E in the total plasma lipoprotein mass (d>1.210 g/ml fraction) of aging rats.

The gradient gels of the HDL subfractions were also densitometrically scanned and only the apo-E (p<0.037) and apo-AI (p<0.054) concentrations of the RII fraction were significantly increased due to copper deficiency (Table 4). No significant increases were observed
Table 4. Effect of Dietary Copper on Apolipoprotein Concentrations in Plasma HDL and Subfractions

<table>
<thead>
<tr>
<th>Sub-fraction</th>
<th>Apolipoprotein</th>
<th>Copper adequate (n=3)</th>
<th>Copper deficient (n=4)</th>
<th>P-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/100 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL</td>
<td>Total protein</td>
<td>83.5 ± 11.1</td>
<td>142.2 ± 5.6</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
<td>4.1 ± 2.2</td>
<td>17.5 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo-AIV</td>
<td>2.9 ± 0.6</td>
<td>4.2 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo-E</td>
<td>8.4 ± 2.2</td>
<td>24.0 ± 4.2</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Apo-AI</td>
<td>57.8 ± 10.9</td>
<td>78.7 ± 8.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Albumin</td>
<td>6.0 ± 1.7</td>
<td>15.6 ± 7.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Total protein</td>
<td>62.4 ± 11.8</td>
<td>88.9 ± 9.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo-AIV</td>
<td>1.3 ± 0.7</td>
<td>1.7 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo-E</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Apo-AI</td>
<td>60.8 ± 11.3</td>
<td>87.0 ± 8.8</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Total protein</td>
<td>15.1 ± 1.0</td>
<td>37.3 ± 1.7</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Apo-AIV</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Apo-E</td>
<td>7.4 ± 1.9</td>
<td>19.1 ± 3.4</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Apo-AI</td>
<td>7.7 ± 2.0</td>
<td>18.6 ± 3.4</td>
<td>0.05</td>
</tr>
</tbody>
</table>

¹Mean ± standard error of the mean

²P-values derived from simple T-test

n=no. of observations

NS=non significant

NR=non retained peak

RI=first retained peak

RII=second retained peak
Figure 3. Sodium Dodecyl Sulfate Polyacrylamide Gradient Gel Electrophoresis and Scanning of the HDL Fraction. Approximately 25 mg of HDL protein from control [A] and copper-deficient [B] rats were applied to each gel. A Zeiss model PM6 spektralphotometer scanned the gels at 660 nm at a speed of 20 mm/min.
for any other apolipoproteins of the subfractions. Marcel et al. (1981) observed progressively increasing apo-E levels from the NR to the R_I to the R_II fraction of human HDL. The apo-E concentration represented 0.07%, 0.4% and 14% of the protein contents of the NR, R_I and R_II fractions, respectively. In the control rats, the NR fraction contained no detectable apo-E while the apo-E concentration made up 0.5% and 49% of the protein contents of the R_I and R_II fractions, respectively. Similarly, in copper-deficient rats, apo-E was still not detectable in the NR fraction and the percentages of apo-E remained relatively unchanged at 0.3% for R_I and 50.7% for R_II. Nevertheless, the relative amount of apo-E was increased in the overall HDL fraction. This increment was due mainly to the increased concentration of apo-E in the R_II fraction (Table 4).

In humans, plasma HDL carry about 20% of the blood borne cholesterol and are responsible for the transport of extra hepatic cholesterol to LDL or the liver for rapid uptake and degradation (Schwartz et al. 1978; Stein and Stein 1973). In rats, the plasma HDL normally carry about 75% of the endogenous cholesterol which may contribute to the atherosclerotic resistance of the rat (Mahley and Holcombe 1977).

The HDL can be subfractionated into two metabolically distinct subfractions: (1) HDL with apo-E, which interact with specific receptors of fibroblasts and smooth muscle cells; and (2) HDL without apo-E, which do not interact with specific receptors (Mahley 1981). Weisgraber and Mahley (1980) were able to subfractionate human HDL into
distinct retained and non-retained fractions based on the interaction of the apolipoprotein E with heparin and manganese. The non-retained fraction was identified as the HDL without apo-E while the retained fraction contained apo-E. They determined that the retained fraction of HDL, containing apo-E, was biologically active by its ability to compete with LDL for binding to cell surface receptor sites of fibroblasts. They also found that the HDL with apo-E were larger in diameter (122 ± 17Å) than HDL without apo-E (95 ± 12Å). In addition, the HDL with apo-E contained significantly more cholesterol and less protein that the HDL without E.

The data in this study of rat HDL identified three distinct subfractions: a non-retained fraction and two retained fractions (R_I and R_II). This is similar to the observations of Marcel et al. (1981) in their separation of human HDL. The R_II fraction contained a significant amount of apo-E and should, therefore, be biologically active in binding receptors. Although the R_I fraction was retained on the heparin-Sepharose column, it contained only trace amounts of apo-E. Marcel et al. (1981) suggests that the retention of R_I may be due to a secondary affinity process: the original ligand, heparin, binds apo-E and/or apo-E containing lipoproteins, which become a secondary ligand; these bound apo-E molecules have an affinity for certain HDL particles such as R_I, thus resulting in their retention on the column. In copper-deficient rats, theapo-E-rich HDL contained significantly less protein and more cholesterol compared to apo-E-poor HDL. The size of the HDL particle was not considered in this study.
Cholesterol feeding has been shown to induce increased concentrations of HDL with apo-E (HDLc) in many species (Mahley 1978). HDLc are cholesterol-rich lipoproteins formed in the plasma or extracellular space from nascent HDL which have acquired apolipoprotein E and have become enlarged by enrichment in cholesterol esters (Mahley 1983). Free cholesterol is esterified by the plasma enzyme lecithin: cholesterol acyltransferase (LCAT) and this cholesterol ester appears to be transferred between HDL and VLDL with apo-E containing HDL acting as equilibrators (Marcel et al. 1981). HDL with apo-E are rapidly cleared from the circulation by the liver through a high affinity, receptor-mediated process. It has been speculated that HDL with apo-E are antiatherogenic and are involved in the reverse transport of cholesterol away from peripheral tissues to the liver for degradation and excretion (Mahley 1981). Hui et al. (1981) recently discovered a unique hepatic apo-E receptor in mature animals capable of interacting specifically with HDL with apo-E, which emphasized the importance of apo-E containing HDL in the pathway of hepatic cholesterol degradation. In addition, the expression of apo-B,E receptors was found to decrease with increasing age while apo-E receptor expression remained constant, demonstrating the important role the apo-E receptor plays in lipoprotein metabolism of the aging animal.

Receptor-mediated hepatic uptake involves the interaction of lipoproteins with membrane receptors mediated by apolipoproteins (Mahley 1983). Positively charged residues of lipoproteins associated with apo-B or apo-E are implicated in binding of LDL to receptors.
Selective modification of either lysine or arginine residues (Mahley 1983) was shown to impair the binding activity of lipoproteins containing apo-B or apo-E. Furthermore, amino acid and sequence analyses of apo-E revealed that major isoforms of apo-E differed from one another by single amino acid substitutions of cysteine or arginine which accounted for relative charge differences observed between the isoforms. These changes in overall surface protein charge affected receptor binding activity to apo-B,E receptors. In addition, studies by Filipovic et al. (1979) have indicated that the sialic acid content of lipoproteins may influence binding activity by changing the net surface charge of the lipoprotein. However, others (Attie et al. 1979) have failed to show the effect of sialic acid on receptor binding of lipoproteins. They observed that desialylation of LDL by neuraminidase (which cleaves the N-acetylneuramic acid terminus of apolipoproteins) did not alter catabolism by hepatic or extra-hepatic cells. Still, the surface charge distribution of lipoproteins may be one of the controlling factors for specific uptake and degradation of lipoproteins by the liver. Copper deficiency may possibly alter arginy1 and sialy1 components of apo-E, during posttranslational modification, which may influence the half-life of the HDL with apo-E by altering hepatic uptake.

The data observed in this study indicate that copper deficiency resulted in an increased concentration of apo-E-rich HDL. It appears that the hypercholesterolemia induced by copper deficiency stimulated apo-E synthesis, thereby increasing the plasma level of apo-E-rich HDL. Although HDL with apo-E have been shown to be efficiently and rapidly
cleared from the circulation, hypercholesterolemia and a prolonged half-life of cholesterol carried by HDL were still observed in copper deficient rats (Lei and Lin 1981). The increase in HDL with apo-E may have resulted from a reduction in their clearance by the receptor-mediated uptake and degradation process. Copper deficiency may have altered the structure of apolipoprotein E and/or the function of lipoprotein receptors and subsequently impaired lipoprotein-receptor binding which resulted in hypercholesterolemia.
SUMMARY

Twenty-four male weanling rats were used in a 7-week experiment to determine the effect of dietary copper deficiency on the lipid, protein and apolipoprotein concentrations of HDL and its subfractions.

Heart weight and heart-to-body-weight ratio were significantly elevated while final body weight, body weight gain, and liver copper content were significantly reduced in rats fed the copper-deficient diet. Protein and cholesterol concentrations of HDL and its R\text{II} subfraction were significantly increased in copper-deficient rats compared to controls. No effect of copper deficiency was observed on triglyceride levels of HDL or its subfractions. The apolipoprotein E concentration of HDL and its R\text{II} subfraction was significantly increased in copper-deficient rats. In addition, copper deficiency significantly elevated the level of apo-AI of the R\text{II} subfraction.

These results and data derived from previous studies suggest that the hypercholesterolemia associated with copper deficiency was due to an impairment in the cholesterol degradation process, regardless of an increase in the apo-E-rich HDL fraction. This may possibly involve an alteration in the uptake of HDL by hepatic receptors.
APPENDIX A

VITAMIN AND MINERAL COMPOSITION

Table 1. AIN-76\textsuperscript{TM} Vitamin Mixture.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Mg per kg mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamin·HCl</td>
<td>600</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>600</td>
</tr>
<tr>
<td>Pyridoxine·HCl</td>
<td>700</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>3000</td>
</tr>
<tr>
<td>D-Calcium Pantothenate</td>
<td>1600</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>200</td>
</tr>
<tr>
<td>d-Biotin</td>
<td>20</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>1</td>
</tr>
<tr>
<td>Retinyl Acetate</td>
<td>800</td>
</tr>
<tr>
<td>dl-\textsuperscript{Tocopheryl Acetate}</td>
<td>5000</td>
</tr>
<tr>
<td>Cholecalciferol</td>
<td>2.5</td>
</tr>
<tr>
<td>Menaquinone</td>
<td>5</td>
</tr>
<tr>
<td>Glucose, finely powdered to make</td>
<td>1000 g</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Per kg mixture</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>CaHPO₄</td>
<td>500 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>74 g</td>
</tr>
<tr>
<td>K₃C₆H₅O₇·7H₂O</td>
<td>220 g</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>52 g</td>
</tr>
<tr>
<td>MgO</td>
<td>24 g</td>
</tr>
<tr>
<td>FeC₆H₅O₇</td>
<td>6 g</td>
</tr>
<tr>
<td>MnCO₃</td>
<td>3200 mg</td>
</tr>
<tr>
<td>ZnCO₃</td>
<td>1600 mg</td>
</tr>
<tr>
<td>KIO₃</td>
<td>10 mg</td>
</tr>
<tr>
<td>Na₂SeO₃·5H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>CrK(SO₄)₂·12H₂O</td>
<td>550 mg</td>
</tr>
<tr>
<td>Glucose, finely powdered to make</td>
<td>1000 g</td>
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</tbody>
</table>
REFERENCES


