The Effect of Copper Deficiency on Rat Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity

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The effect of copper deficiency on hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme regulating cholesterol biosynthesis, was investigated in the rat. Male weanling rats were fed semipurified diets containing adequate, marginal, or deficient levels of copper for 6 weeks. Two separate studies were conducted; in the first study, animals were fasted 12 hours prior to analysis and in the second study, animals were fed diets ad libitum. Plasma lipid levels, hepatic cholesterol concentrations, and 3-hydroxy-3-methylglutaryl coenzyme A reductase specific activity, total and active, were determined. Consistent with previous findings, plasma total cholesterol and triglyceride levels were significantly elevated in copper-deficient rats. Copper deficiency resulted in a significant decrease in hepatic total cholesterol levels. Total and active levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase in fed animals were elevated twofold with copper deficiency, with the active form of the enzyme constituting approximately 30% of total activity. 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity in copper-deficient fasted rats was twofold higher than for the fasted adequate animal; however, fasting did result in a 10-fold reduction in hepatic reductase specific activity. These data support the hypothesis that copper deficiency results in a hypercholesterolemic state in the rat associated with increased hepatic cholesterol synthesis.

Keywords: Cholesterol; copper deficiency; HMG-CoA reductase; rat; hypercholesterolemia

Introduction

Copper has been shown to be an essential mineral for all species studied to date. Although copper deficiency is rare in adult humans, its symptoms have been diagnosed in individuals suffering from the genetic disorder Menke's disease,[1] in patients on total parental nutrition lacking copper,[2] and during the rehabilitation of malnourished children.[2] Although clinical deficiencies of copper are rare, recent data indicate that many individuals may suffer from marginal inadequacies in copper status because they receive less than the 2.0 mg/d copper that has been established as necessary.[3,4]

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In the rat, some of the earliest signs of copper deficiency, along with anemia,[5] are elevated levels of plasma cholesterol[6-10] and triglyceride.[11,12] This phenomenon was first observed in 1973 when Klevay and coworkers[6] found an elevated plasma cholesterol in copper-deficient rats when the dietary zinc to copper ratio was increased. This increase in plasma cholesterol was attributed to an increased zinc to copper ratio since it is known that zinc and copper will compete for absorption in the gut.[13,14] Shortly after this observation was reported, several large-scale factorial studies by Murthy and colleagues,[15,16] indicated that serum cholesterol and copper levels were inversely related, while no relationship was found between zinc and serum cholesterol levels. In 1977, Lei[9] confirmed that a deficiency of copper alone resulted in elevated plasma cholesterol and triglyceride levels in rats.

In an attempt to explain how copper deficiency may be linked to an elevated plasma cholesterol level, hepatic cholesterol metabolism and its regulation have been examined in copper-deficient animals to deter-

mine whether abnormalities exist. In 1977, Lei[9] incubated rat liver slices from copper-adequate and copper-deficient rats with [^{14}C]acetate and found that copper status had no effect on the rate of incorporation of ^{14}C into cholesterol. In 1980, Shao and Lei[17] injected rats with [^{14}C]mevalonate and found an increase in [^{14}C]cholesterol ester in plasma from copper-deficient animals. This finding suggested that there is a shift of hepatic cholesterol to the plasma. To examine whether or not cholesterol catabolism was altered by copper deficiency, Lei[18] measured rates of bile acid synthesis and fecal cholesterol excretion in copper-deficient rats and found no difference compared with control animals. Furthermore, Klevay and colleagues[19] demonstrated that the excretion of fecal acidic sterols was not impaired in copper-deficient rats. These studies imply that the observed hypercholesterolemia in copper-deficient rats may not be the result of altered cholesterol excretion and suggest that increased cholesterol synthesis may be a contributing factor.

The present study has reexamined this question by measuring the activity of the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (E.C.1.1.1.34), in copper-adequate and copper-deficient rats. The data demonstrate that with copper deficiency, HMG-CoA reductase activity is significantly elevated, suggesting that increased cholesterol synthesis may account in part for the hypercholesterolemia observed in copper deficiency.

Materials and Methods

Materials

Materials were obtained from the following sources: DL-hydroxy-[3-^{14}C]methylglutaryl coenzyme A (40 to 60 mCi/mmol) and DL-[5-^{3}H]mevalonic acid (10 to 30 Ci/mmol), New England Nuclear, Boston, MA, USA; cholesterol enzymatic assay kit and cholesterol esterase, Boehringer Mannheim, Indianapolis, IN, USA; triacylglycerol enzymatic assay kit, DL-3-hydroxy-3-methylglutaryl coenzyme A, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and βNADPH, Sigma, St. Louis, MO, USA; and silica gel thin layer chromatography plates, Alltech Associates, Deerfield, IL, USA.

Animals

Three-week-old weanling male Sprague-Dawley rats weighing approximately 55 g were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN, USA. Animals were housed individually in stainless steel cages in a light-cycle room with 7:00 AM to 7:00 PM light.

Diets

The animals were divided randomly into two or three dietary treatment groups, depending on the study, and maintained for 6 weeks on the semipurified diets described in *Table 1*. Dietary copper levels were determined after a nitric acid digestion by flame atomic absorption spectrophotometry (model 170, Hitachi, Tokyo, Japan).

Table 1 Diet composition[a]

Component	Percent (wt/wt)		
	Adequate	Marginal	Deficient
Casein	20.0	20.0	20.0
DL-methionine	0.3	0.3	0.3
Glucose monohydrate	65.0	64.5	64.0
Cellulose	5.0	5.0	5.0
Corn Oil	5.0	5.0	5.0
AIN mineral mix[b]	3.5	3.5	3.5
AIN vitamin mix	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2
Cupric carbonate	1.0	0.5	0.0
Copper Level (ppm)			
Study 1	6.55	1.64	0.89
Study 2	6.92	—	0.57

[a] Diets were prepared according to specifications provided by the American Institute of Nutrition,[20] with the exception that copper was not included in the mineral mix. Copper levels in each diet were determined by analysis on an atomic absorption spectrophotometer.
[b] AIN Mineral mix[20] contains standard contents with the exception of copper.

Study design

Two separate studies were performed. The first study (study 1—fasting) used 18 rats that were randomly assigned to three dietary treatments which were identical except for varying copper levels: adequate (6.55 ppm), marginally deficient (1.64 ppm), and deficient (0.89 ppm). Groups of six animals were given their respective diet ad libitum for 6 weeks and were fasted for 12 hours prior to death. The second study (study 2—fed) involved 12 rats randomly divided into two dietary groups, copper-adequate (6.92 ppm) and copper-deficient (0.57 ppm). Animals in this study were not fasted prior to death. Dietary copper levels for studies 1 and 2 are presented in *Table 1*. This design allowed for comparison of fasting to non-fasting effects on HMG-CoA reductase activity in response to varying levels of dietary copper.

Assays

Plasma cholesterol, triglyceride, and hematocrit. Blood was obtained by cardiac puncture using Na_2EDTA (1 mg/ml) as an anticoagulant. Following centrifugation, plasma was removed and total cholesterol and triglyceride were determined by enzymatic assay.[21,22] Hematocrit was determined by centrifugation in a capillary tube system to obtain packed cells.

Hepatic microsome isolation and HMG-CoA reductase assay. Animals were killed between 7:00 to 9:00 AM near the nadir of the diurnal rhythm for HMG-CoA reductase.[23] At the time of death, livers were quickly excised, rinsed with saline, and placed in ice-cold Dul-

becco's phosphate buffered saline (pH 7.2). A crude homogenate was prepared by pressing tissue through a tissue grinder into 1/3 (wt/vol) homogenization buffer (50 mM KH_2PO_4, 0.1 M sucrose, 50 mM KCl, 30 mM EDTA, and 2.0 mM dithiothreitol; pH 7.2) containing either 50 mM NaCl, to determine total HMG-CoA reductase activity, or the phosphatase inhibitor NaF (50 mM) for measurement of the active form of enzyme. This crude homogenate was further homogenized with a Potter-Elvehjem homogenizer. A microsomal fraction was isolated by two 15-minute centrifugations at 10,000 × *g* followed by a 1-hour centrifugation at 100,000 × *g* at 4°C. Microsomes were rehomogenized in a small volume of homogenization buffer and stored at −70°C.[23]

The assay for HMG-CoA reductase was essentially the radioisotopic thin-layer chromatography method described by Shapiro et al.[24] Briefly, our method used up to 0.20 mg protein per assay mixture which contained 50 nmol RS-[^{14}C]HMG-CoA, 4.5 μmol glucose-6-phosphate, 3.6 μmol EDTA, 0.45 μmol βNADPH, 0.3 IU glucose-6-phosphate dehydrogenase, and 2 × 10^4 dpm [^{3}H]mevalonic acid brought to a final volume of 100 μl with homogenization buffer. Incubations were carried out for 15 minutes at 37°C, and the [^{14}C]-mevalonolactone isolated by thin layer chromatography on silica gel plates was developed in benzene/acetone (vol/vol; 1/1).[24] The R_f for mevalonolactone was identified by spotting [^{3}H]mevalonolactone and determination of radioactivity for each centimeter of the silica gel plate. Microsomal protein concentrations were determined by the method of Markwell et al.[25] Enzyme-specific activities are expressed as picomoles of mevalonate produced per minute per milligram microsomal protein.

Hepatic cholesterol concentrations. Approximately 1.0 g of liver from each rat was homogenized in a Potter-Elvehjem homogenizer with an equal volume of Dulbecco's phosphate buffered saline. The homogenate was extracted with 10 ml of chloroform/methanol(2/1; vol/vol).[26] To remove tissue precipitates, extracts were filtered on no. 1 grade filter paper and the filter paper was washed with approximately 10 ml additional solvent. Samples were brought to equal volume and aliquots in quadruplicate were removed to dry overnight.

To assay for total and free cholesterol, a modification of the assay procedure described by Sale et al.[27] was used. Ethanol (0.2 ml) was added to the dry samples followed by 0.8 ml of a buffered solution containing horseradish peroxidase (0.2 U/ml), cholesterol oxidase (0.125 U/ml), and O-dianisidine (0.1 mg/ml). Samples were incubated for 10 minutes at 37°C, then 100 mU of cholesterol esterase were added to half of the tubes. Incubation was allowed to proceed for another 35 minutes at 37°C. Absorbance at 500 nm was determined and milligrams per gram of cholesterol were calculated relative to a standard curve. Cholesterol ester values were determined as the difference between the cholesterol oxidase reaction values (free cholesterol only) and values obtained from the reaction containing cholesterol oxidase and cholesterol esterase (total cholesterol levels).

Statistics

One-way analysis of variance was used to assess differences between treatment groups. For determinations of differences in plasma cholesterol levels, a one-tailed Stuent's *t* test was used since it was predicted that plasma cholesterol would increase with copper deficiency. All data are presented as mean ± 1 SD.

Results

To verify that animals fed the copper-deficient diet were indeed copper-deficient, several parameters characteristic of copper deficiency were determined (*Table 2*). Copper-deficient rats had decreased body weights and hematocrits, and exhibited increased heart weights and heart to body weight ratios. Also, in the study 2—fed group, liver weights were significantly different. All of these traits are characteristic of

Table 2 Characteristics of copper deficiency

	Body weight (g)	Liver weight (g)	Heart weight (g)	Heart/body weight (%)	Hematocrit (%)
Study 1—fasting					
Adequate	326 ± 16^a	9.07 ± 0.4	1.15 ± 0.03^b	0.35 ± 0.01^c	50.1 ± 2.1^a
Marginal	289 ± 44^b	9.62 ± 0.9	1.36 ± 0.09^a	0.47 ± 0.05^b	40.4 ± 2.7^b
Deficient	238 ± 22^c	8.86 ± 0.2	1.45 ± 0.09^a	0.61 ± 0.02^a	27.7 ± 1.7^c
	($P < .001$)		($P < .001$)	($P < .001$)	($P < .001$)
Study 2—fed					
Adequate	303 ± 9^a	13.0 ± 1.2^a	1.07 ± 0.06^b	0.35 ± 0.02^b	49.2 ± 1.5^a
Deficient	219 ± 25^b	10.1 ± 1.7^b	1.77 ± 0.39^a	0.81 ± 0.17^a	28.1 ± 2.2^b
	($P < .001$)	($P < .01$)	($P < .01$)	($P < .001$)	($P < .001$)

Male weanling rats (18 animals, study 1—fasting; 12, study 2—fed) were fed diets containing either adequate, marginal, or deficient levels of copper for a period of 6 weeks. At death, several parameters of copper status were determined. Values in the same column with differing superscripts indicate significantly different values.

Table 3 Plasma lipids

	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Study 1—fasting		
Adequate	109.0 ± 3.5^{b}	99.8 ± 11.4^{b}
Marginal	120.2 ± 17.6^{ab}	114.0 ± 9.5^{b}
Deficient	137.4 ± 24.0^{a}	202.9 ± 104^{a}
	($P < .05$)	($P < .05$)
Study 2—fed		
Adequate	125.1 ± 8.9^{b}	ND
Deficient	156.9 ± 19.5^{a}	ND
	($P < .01$)	

Rats were fed semisynthetic diets containing either deficient, marginal, or adequate levels of copper. At death, whole blood was obtained by cardiac puncture using EDTA as an anticoagulant. Plasma was separated from blood by centrifugation. Total plasma cholesterol and triglyceride concentrations were determined by enzymatic assay as previously described. Study 1: n = 18, 12-hour fasted rats. Study 2: n = 12, non-fasted rats. The data are presented as means ± SD, ND = not determined. Values in the same column with differing superscripts are significantly different.

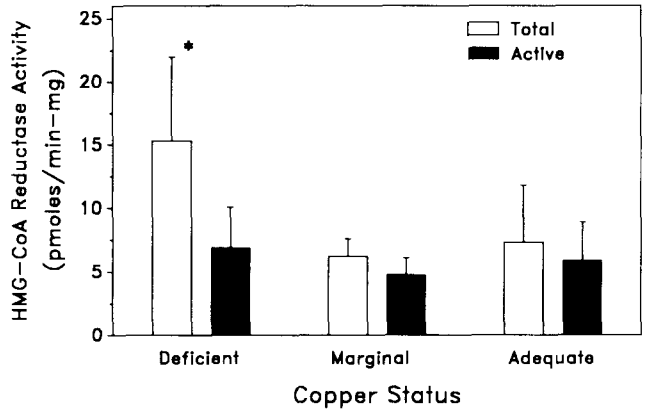

Figure 1 Rats were fed copper-containing diets as indicated in Table 1 (study 1—fasting) with either adequate (n = 6), marginal (n = 6), or deficient (n = 6) levels of copper for 6 weeks. Prior to death, animals were fasted for 12 hours. Hepatic microsomal fractions were isolated and HMG-CoA reductase total and active specific activities were determined. Total hepatic HMG-CoA reductase activity was significantly greater for deficient rats than for adequate animals (*$P < .01$).

copper deficiency and indicate that animals on the copper-deficient diets were copper-deficient.

Similar to previous studies,[6-12] we observed both hypercholesterolemia and hypertriglyceridemia in copper-deficient rats (*Table 3*). Plasma cholesterol levels in copper-deficient rats were 25% greater than for copper-adequate animals in both the fasted and fed state. Triglycerides in fasted rats were twofold greater than those observed in copper-adequate rats.

Analysis of rat liver microsomal HMG-CoA reductase in copper-deficient rats showed a net twofold increase in total HMG-CoA reductase activity relative to copper-adequate animals for both the fasted and fed animals (*Figures 1* and *2*). In the fed copper-deficient animals, the active form of HMG-CoA reductase was also elevated twofold; however, the active form of the enzyme was not changed in the fasted rats. Fasting resulted in a 10-fold reduction in HMG-CoA reductase activity for both total and active forms of the enzyme (*Figure 1*). In fasted rats, the observed active to total enzyme ratio was 45% for copper-deficient rats and ranged from 75% to 80% for the marginal and adequate groups (*Figure 1*). In the fed state, the active form of HMG-CoA reductase was approximately 30% for both copper-adequate and copper-deficient rats (*Figure 2*).

Copper deficiency resulted in decreased total hepatic cholesterol levels for both fasted and fed animals (*Table 4*). Under all conditions, the majority of cholesterol was present as free cholesterol, relative to esterified cholesterol. It is interesting to note that in fasted animals, the concentration of free cholesterol changes with copper status, whereas in fed animals,

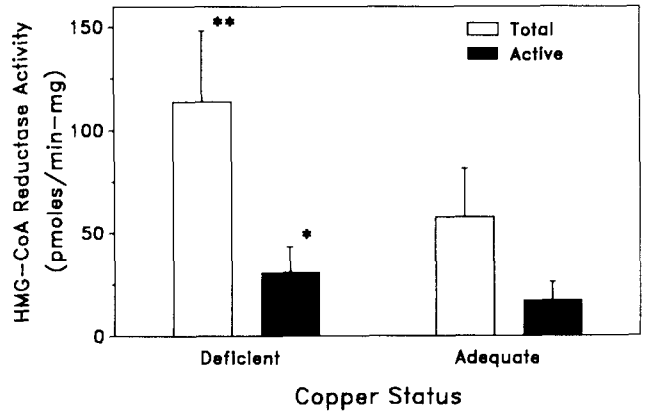

Figure 2 Rats were fed diets containing either adequate (n = 6) or deficient (n = 6) levels of copper as indicated in Table 1 (study 2—fed) for a period of 6 weeks. Animals were fed ad libitum prior to death. Hepatic microsomal fractions were isolated and HMG-CoA reductase specific activities, both total and active, were determined. Total and active forms of HMG-CoA reductase were significantly greater for deficient than adequate rats (*$P < .05$; **$P < .01$).

Table 4 Hepatic tissue cholesterol levels

	Cholesterol (mg/g)		
	Total	Free	Ester
Study 1—fasting			
Adequate	2.12 ± 0.16^{a}	1.79 ± 0.07^{a}	0.33 ± 0.14
Marginal	1.92 ± 0.41^{ab}	1.62 ± 0.42^{ab}	0.32 ± 0.19
Deficient	1.57 ± 0.16^{b}	1.26 ± 0.32^{b}	0.32 ± 0.30
	($P < .05$)	($P < .05$)	
Study 2—fed			
Adequate	1.64 ± 0.10^{a}	1.35 ± 0.13	0.29 ± 0.06^{a}
Deficient	1.47 ± 0.08^{b}	1.32 ± 0.13	0.17 ± 0.11^{b}
	($P < .001$)		($P < .01$)

Rats were fed semisynthetic diets as described with either adequate, marginal, or deficient levels of copper. At death, approximately 1.0 g of liver was removed and stored at −20°C. Hepatic lipids were extracted using chloroform methanol, and free and total levels of cholesterol were determined by enzymatic assay. Studies 1 and 2 are as described for Table 3. Data are presented as means ± SD; n = 6 animals per group. Values in the same column with differing superscripts are significantly different.

esterified cholesterol levels are reduced with copper deficiency while free cholesterol remains essentially constant. The percentage of cholesterol present as cholesterol ester increases from 15.6% to 20.4% with copper deficiency in the study 1—fasting group; however, this trend is reversed in the study 2—fed group. None of these percentage changes are significant for either group.

Discussion

As previous investigators have established, we observed an elevated plasma total cholesterol for both fed and fasted copper-deficient rats.[6-10] Our marginally deficient group in study 1 had a somewhat elevated plasma cholesterol intermediate between the adequate and deficient groups. Also, consistent with previous reports, we observed an elevated plasma triglyceride in fasted copper-deficient rats.[11,12] Triglycerides were not determined in fed animals due to post-prandial fluctuations in plasma triglyceride levels.

Our findings of an increased HMG-CoA reductase with copper deficiency support the hypothesis that copper deficiency results in increased rates of hepatic cholesterol biosynthesis, since hepatic HMG-CoA reductase activity generally reflects hepatic cholesterol synthesis.[9] In both fed and fasted copper-deficient rats, HMG-CoA reductase activity was elevated twofold, which suggests that an elevation of cholesterol biosynthesis may be a physiologic manifestation of copper deficiency. Previous investigators may not have observed this elevation of cholesterol synthesis with copper deficiency because in many cases fasted rats were used,[9,18] and it is well-established that HMG-CoA reductase and cholesterol biosynthesis are markedly reduced under fasting conditions.[28-30] This is consistent with our own data for the fasted rat which showed a 10-fold reduction in HMG-CoA reductase activity in the fasted state. It is also possible that previous studies[9,18] did not observe elevated cholesterol synthesis with copper deficiency due to technical problems associated with using radiolabeled acetate, a substrate subject to precursor pool variations.[31] This artifact makes determination of precursor-specific activity difficult and comparison of incorporation rates ambiguous.

Previous investigators have shown that hepatic HMG-CoA reductase exists *in vivo* in two distinct forms, a phosphorylated inactive form and a dephosphorylated active form.[32,33] The interconversion between these two forms has been shown to be mediated by a phosphatase and a series of kinases that are themselves subject to regulation.[34-36] In general, the percentage of active to total levels of HMG-CoA reductase for the chow-fed rat has been found to range between 15% to 30% under normal conditions.[37,38] It is interesting to note that although copper deficiency dramatically altered total levels of HMG-CoA reductase activity, copper deficiency did not alter the ratio of active to total enzyme in the fed animal, which was 30% for both copper-adequate and copper-deficient rats. This suggests that the observed increase in reductase activity with copper deficiency is not due to an activation of the 70% of reductase which is inactive. Instead, it suggests that copper deficiency results in a net increase in the total levels of HMG-CoA reductase. This would be in agreement with the theory that alterations in the ratio of phosphorylated to dephosphorylated enzyme function primarily as a short-term, transient mechanism for regulation of reductase,[39] whereas changes in enzyme levels are important for more long-term adaptations to environmental changes.

In the fasted rat, we also observed a net twofold increase in HMG-CoA reductase activity with copper deficiency; however, in the fasted state, the ratio of active to total HMG-CoA reductase was increased to 50% to 80%. There are several possible explanations for why this may occur. First, under fasting conditions, HMG-CoA reductase activity was repressed to nearly 10% of fed levels, and it is possible that when the enzyme is suppressed to this extent, the active form of the enzyme becomes a larger percentage of total activity.[40] Also, under these circumstances, in which activity levels are very low, it may be analytically impossible to distinguish between the inactive-phosphorylated and active-dephosphorylated form of reductase.

Previous studies have consistently shown a decreased hepatic cholesterol content in copper-deficient rats.[9,12] Similarly, our studies found significantly reduced levels of total hepatic cholesterol in both fed and fasted animals. This finding implies a possible mechanism by which hepatic HMG-CoA reductase may be induced during copper deficiency. HMG-CoA reductase has been shown to be responsive to many regulatory factors, including hormones[39] and feedback inhibition by biosynthetic intermediates;[41] however, one of the primary regulatory factors governing the overall activity of this enzyme has consistently been shown to be hepatic cholesterol levels.[42] Thus, the predicted response to a reduced hepatic cholesterol level would be, as observed, an elevation in reductase activity.

Our findings, together with other data, support the hypothesis that the hypercholesterolemia induced by copper deficiency results from a combination of factors that include increased rates of export of newly synthesized cholesterol from the liver,[17] increased rates of cholesterol ester and apoprotein uptake by extrahepatic tissues,[43,44] and increased levels of cholesterol synthesis coupled with unchanging rates of bile acid synthesis[18] and cholesterol excretion.[19] An additional factor that may effect plasma cholesterol homeostasis is the recent observation that copper deficiency decreases endothelial lipoprotein lipase and hepatic lipase activity in the rat which could contribute to an elevated plasma cholesterol.[45] Considered together, these observations support the hypothesis that hypercholesterolemia is due to increased hepatic synthesis and export of cholesterol with no alterations in cholesterol excretion.

Our findings suggest that the mechanism by which

copper deficiency results in elevated plasma cholesterol may partly be due to increased levels of hepatic HMG-CoA reductase and endogenous cholesterol synthesis. The mechanism by which HMG-CoA reductase is elevated in copper deficiency may relate to the reduction in hepatic cholesterol levels. The mechanism by which hepatic tissue cholesterol concentrations are reduced with copper deficiency probably results from a combination of factors that leads to a general shift in cholesterol distribution between the plasma and hepatic compartments; however, the mechanisms by which copper deficiency facilitates this change in cholesterol distribution are not yet fully understood.

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