Copper Deficiency Increases *In Vivo* Hepatic Synthesis of Fatty Acids, Triacylglycerols, and Phospholipids in Rats (43640)

ABDULLAH A. AL-OTHMAN,[1] FUMIKO ROSENSTEIN, AND KAI Y. LEI[2]

Department of Nutrition and Food Science and Nutritional Sciences Program, University of Arizona, Tucson, Arizona 85721

Abstract. This study examined the influence of dietary copper status on the *in vivo* hepatic fatty acid synthesis and the incorporation of nascent fatty acids into various hepatic lipid classes. Fifty weanling male Sprague-Dawley rats were assigned to two dietary treatments, copper deficient (5.4 nmol/g of diet) and copper adequate (102 nmol/g of diet). After 7 weeks of treatment, rats were injected with 0.111 MBq of [1-^{14}C] acetate (1.85 GBq/m*M*)/100 g body wt through the femoral vein. Five rats from each treatment were sacrificed at 3, 6, 9, 12, and 24 min after injection. Radioactivities of nascent fatty acid samples were used to determine relative rates of fatty acid synthesis and their assembly into triacylglycerols and phospholipids. Linear increases were observed up to 12 min after injection for total hepatic fatty acid synthesis and their assembly into triacylglycerols and phospholipids for both treatments. In addition, 46% and 30% of total fatty acid synthesized were assembled into triacylglycerols and phospholipids, respectively, for both groups. Furthermore, hepatic fatty acid synthesis and assembly into triacylglycerols and phospholipids were enhanced more than 2-fold by copper deficiency when the data were expressed as per liver per 100 g body weight. [P.S.E.B.M. 1993, Vol 204]

Hypercholesterolemia induced by copper deficiency has been observed in many species, including humans (reviewed in [1]). In addition, alterations in plasma lipoprotein profiles induced by Cu deficiency have been reported by numerous investigators (reviewed in [2]). Comprehensive data on the concentration, pool size, and percentage of composition for the lipid and protein components of major lipoprotein classes in Cu-deficient rats were reported recently by Al-Othman *et al.* (3). Marked increases in triacylglycerol percent composition of lipoproteins, particularly that of very low density lipoproteins, have been observed in Cu-deficient rats (3) and hamsters (4). These may have resulted from a reduction in lipoprotein lipase activity, an enhanced hepatic lipogenesis, or both. Although reductions in post-heparin plasma lipoprotein lipase activity have been reported in Cu-deficient rats (5, 6), the significance of these findings is uncertain because the enzyme activity measured in plasma was not corrected for the enlargement of plasma volume usually associated with Cu deficiency. Thus, an increase in hepatic lipogenesis may contribute to the elevation of plasma triacylglycerol level observed in Cu-deficient rats. Indeed, a 2-fold increase in the capacity of rat liver slices to synthesize fatty acids was reported in an early *in vitro* study (7). However, an increase in fatty acid synthesis and the assembly of nascent fatty acids into triacylglycerols and phospholipids has not been demonstrated *in vivo*.

The present study was designed to establish for the first time that Cu deficiency induces an increase in *in vivo* hepatic fatty synthesis and in the subsequent incorporation into triacylglycerols and phospholipids.

Methods

Experimental Design. Fifty weanling male Sprague-Dawley rats, 3 weeks old and weighing 35–40 g, were randomly divided into two dietary Cu treatments: de-

[1] Present address: Department of Food Science and Human Nutrition, King Saud University, Riyadh 11451, Saudi Arabia.

[2] To whom requests for reprints should be addressed at Department of Nutrition and Food Science, 309 Shantz Building, University of Arizona, Tucson, AZ 85721.

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ficient (5.4 nmol/g diet) and adequate (102 nmol/g diet). Rats were housed individually in stainless steel cages in a room maintained at 22°C with 12 hr each of light and darkness (1800 to 0600 hr). Body weights were recorded weekly throughout the experiment. Diets and distilled, demineralized water were provided *ad libitum*. This study was approved by the institutional animal care and use committee.

Experimental Diet. The basal diet (Cu deficient) was similar to that recommended by the American Institute of Nutrition (8), except that no Cu supplement was included in the mineral mix (Table I). The Cu-deficient diet contained 5.4 nmol (0.34 μg) of Cu/g of diet, as measured by atomic absorption spectrophotometry. Cu carbonate was added to the basal diet to provide the Cu-adequate diet with 102 nmol (6.5 μg) of Cu/g of diet.

Determination of in vivo *fatty acid synthesis. In vivo* estimates of fatty acid synthesis were obtained by using [1-^{14}C]acetate (9, 10). After 7 weeks of treatment, feed was removed at 2000 hr for 10 hr and rats were anesthetized with sodium pentobarbital. Five rats were randomly selected from each dietary treatment and assigned to be sacrificed at 3, 6, 9, 12, and 24 min after [1-^{14}C]acetate injection. [1-^{14}C]Acetate (0.111 MBq/100 g body wt; 1.85 GBq/mmol; Amersham Corp., Arlington Heights, IL) was injected via the femoral vein as a solution in 0.33 ml of 0.9% NaCl (9). At specified times, rats were further anesthetized with ether and sacrificed. Rats were completely anesthetized with ether at 1 min before the specified times. The thoracic cavity was opened, exposing the heart. Blood was collected exactly at specified times by cardiac puncture and plasma was obtained by centrifugation. Hematocrit was determined and plasma volume was calculated by the formula established by Carr and Lei (11) for Cu-deficient and -adequate rats. Liver was excised, weighed, frozen in liquid nitrogen, and stored at −80°C.

Table I. Diet Composition

Ingredient	Cu adequate[a]	Cu deficient[a]
Casein	20.0	20.0
DL-Methionine	0.3	0.3
Glucose monohydrate	64.0	65.0
Cellulose (fiber)	5.0	5.0
Corn oil	5.0	5.0
AIN mineral mix[b]	3.5	3.5
AIN vitamin mix	1.0	1.0
Choline bitartrate	0.2	0.2
Cupric carbonate (1.05 g/kg) mix	1.0	0.0
	100.0	100.0
Dietary Cu (nmol/g)[c]	102.0	5.4

[a] Data are expressed as the percentage of composition.
[b] Contains all minerals except copper.
[c] Dietary Cu was determined by atomic absorption spectrophotometry.

Lipid extraction. Total hepatic lipids were extracted by using the method of Folch *et al.* (12). Weighted samples of liver, approximately 2 g, were thawed at 4°C and homogenized in 24 ml of ice-cold chloroform-methanol (2:1; v/v) until a fine suspension of liver was achieved by using a Polytron homogenizer (Brinkman Instrument, Westbury, NY). The tissue extraction mixture was allowed to stand overnight in the tightly closed Teflon screw-cap homogenization tube. The homogenate was heated in a shaking water bath at 45°C for 30 min and then filtered. The homogenate in the filter was washed three times with 5 ml of chloroform:methanol (2:1; v/v). Extracts were evaporated to dryness and residues were redissolved in 5 ml of chloroform and stored at −20°C. Hepatic triacylglycerol and phospholipid contents were quantitated colorimetrically by the methods of Fletcher (13) and Bartlett (14), respectively. Liver cholesterol concentrations were determined by the enzymatic method of Roeschlau *et al.* (15).

Determination of ^{14}C incorporations into hepatic total fatty acids. A 2-ml portion of extracted lipids was placed in a 70-ml screw-cap tube and evaporated to dryness under nitrogen at 50°C. Twenty milliliters of 5% potassium hydroxide in 95% distilled methanol were added to the lipids. The tube was closed tightly and placed in a 80–85°C water bath for 3 hr. Then, 20 ml of water were added and the nonsaponifiable fraction of this solution was extracted three times with 5 ml of petroleum ether. After the extraction of the nonsaponifiable fraction, the aqueous layer was acidified with 6 *N* HCl. Total fatty acids were extracted with petroleum ether three times. The combined extract was transferred into a counting vial and dried under nitrogen. The residue was readily solubilized in 10 ml of Aquasol (New England Nuclear, Boston, MA) for scintillation counting. The radioactivity associated with fatty acids was measured by a liquid scintillation system (model 460C; Packard Instrument, Downers Grove, IL).

Determination of ^{14}C distribution in hepatic lipids. Aliquots of the remaining lipid extract (2.5 ml) were dried under nitrogen atmosphere. Dried lipids were redissolved in 0.5 ml of chloroform and applied on silica gel 60 plates (Alltech Associates, Deerfield, IL). Thin layer chromatography was performed according to the method of Skipski and Barclay (16). The developing system was a mixture of hexane:ether:acetic acid:methanol (90:20:2:3, v/v). The solvent front was allowed to reach 1 cm from the top of plates. After drying the plate under the fume hood, the location of the spots was determined by spraying with a 0.05% solution of rhodamine in 50% ethanol and viewing under ultraviolet light (17, 18). Standard solution of various lipids was partitioned to identify their locations

on the plate. In this system, phospholipids were located at the spot where sample was applied. In contrast, the cholesteryl esters were found to migrate near the solvent front, followed by triacylglycerols, free fatty acids, and free cholesterol. The spots containing the individual lipid classes (phospholipids, triacylglycerols, cholesteryl esters, and free fatty acids) were carefully scraped from the plates and transferred into the test tubes. The silica gel was extracted for lipids with chloroform:methanol (2:1; v/v) three times. For the determination of the radioactivity in free fatty acids, the solution containing free fatty acids was transferred to liquid scintillation vials and evaporated to dryness. Then 10 ml of Aquasol were added. However, solutions of triacylglycerols, phospholipids, and cholesteryl esters were transferred to a 70-ml screw-cap tube and evaporated to dryness. Twenty milliliters of 5% potassium hydroxide in ethanol were added and the lipids were saponified at 80–85°C for 3 hr. The potassium hydroxide solution was diluted with 20 ml of water, and the nonsaponifiable lipids of this solution were extracted three times with 5 ml of petroleum ether. The aqueous phase was acidified with 6 *N* HCl, and fatty acids were extracted three times with 5 ml of petroleum ether. The ether was evaporated under a stream of nitrogen, the fatty acids were dissolved in 10 ml of Aquasol scintillation solution, and the sample was measured for radioactivity by liquid scintillation counting (19).

All data were evaluated by analysis of variance. In two-way analysis of variance, individual degrees of freedom were partitioned by orthogonal comparisons for the main effects and their interactions. *P*-values less than 0.05 indicated significance.

Results

Reductions in body weight, hematocrit, liver Cu, and cholesterol concentrations, as well as increases in liver weight to body weight ratio and plasma volume, were observed in rats fed the Cu-deficient diet (Table II). All of these parameters are characteristic of Cu deficiency and indicate that animals fed the Cu-deficient diet were indeed Cu deficient. A small reduction in cholesterol content and no alteration in phospholipid and triacylglycerol contents were observed in the livers of rats fed the Cu-deficient diet (Table II).

The first objective of this study was to determine the influence of dietary Cu on the total fatty acids synthesis. The incorporation of [1-^{14}C]acetate into total hepatic fatty acids (dpm/g liver) was found to be increased linearly with time up to 12 min after injection and dropped to 74% of it's maximal level by 24 min (data not shown). In order to make a valid treatment comparison, results were expressed also as per liver organ, normalized to 100 g body weight. As a result of Cu deficiency, an approximately 1.9-fold increase in the conversion of [1-^{14}C]acetate into hepatic total fatty acids (dpm/liver/100 g body wt) was observed at 9 min after injection (Table III).

The second objective of this study was to determine the types of lipids into which the radioactive fatty acids were incorporated. When the radioactivities of the fatty acids associated with triacylglycerols and phospholipids were expressed as a percentage of total hepatic fatty acid radioactivity, as much as 48.2% and 27.7% were observed for controls and 43.4% and 32.2% were established for deficient rats, respectively (Table IV). In addition, the percentages of the radioactivity of total fatty acids incorporated into cholesteryl esters and free fatty acids were 5.9% and 14.0% for adequate rats and 3.7% and 12.1% for deficient rats, respectively. No significant difference in the percentage of the radioactivity of total fatty acids incorporated into each type of lipids was observed among the two treatments.

The results of the incorporation of ^{14}C-acetate into the liver fatty acids of triacylglycerols and phospholipids after various periods of time are presented in Table V. As early as 3 min after injection, the incorporation of ^{14}C-acetate into fatty acids of triacylglycerols and phospholipids (dpm/liver/100 g body wt) was elevated 2.0- and 2.3-fold, respectively, in rats fed the Cu-deficient diet. The highest incorporation activity was obtained after 12 min of injection for both groups. Therefore, the overall trend of the radioactivity incorporated into triacylglycerols and phospholipids (Table V) appeared to be similar to that observed for the total fatty acids (Table III).

Unlike the other lipid classes, the incorporation of ^{14}C into fatty acids of the hepatic cholesteryl ester and free fatty acids (dpm/g liver) appeared not to be influenced by dietary Cu treatment. However, when the data were expressed as per liver corrected for body weight, the incorporation of ^{14}C-acetate into hepatic-free fatty acids was significantly increased almost 70% at 9 min in Cu-deficient rats (Table VI). For both treatments, a gradual elevation in the incorporation of radioactivity into fatty acids of cholesteryl esters was linear up to 12 min, but that of free fatty acids appeared to have peaked at 9 min after injection.

Discussion

In the present study, the relative rate of hepatic lipogenesis observed in rats fed the adequate diet is consistent with results reported by Sakurai *et al.* (9) in young male rats fed a diet presumably adequate in all nutrients. In rats fed the adequate diet, the distribution of radioactivity of total hepatic fatty acids among triacylglycerols, phospholipids, and cholesteryl esters was 48%, 28%, and 6%, respectively (Table IV). Similar results were observed in arterial lipids labeled with [1-^{14}C]acetate from young pigeons fed a nutritionally adequate diet (18). The percentage of total radioactivity of arterial lipids associated with triacylglycerols, phos-

Table II. Influence of Dietary Copper on Body and Liver Weights, Hematocrit, Plasma Volume, Liver Copper, Triacylglycerol, Phospholipid, and Cholesterol Concentrations[a]

	Cu adequate	Cu deficient	ANOVA[b]
Body wt (g)	274 ± 5	204 ± 7	<0.001
Liver wt (g/100 g body wt)	3.65 ± 0.14	5.24 ± 0.10	<0.001
Hematocrit (% packed cell volume)	51.20 ± 1.05	21.90 ± 1.03	<0.001
Plasma volume (ml/100 g body wt)	3.77 ± 0.06	6.43 ± 0.13	<0.001
Liver copper (nmol/g wet wt)	62.6 ± 3.6	12.3 ± 3.0	<0.001
Liver triacylglycerols (μmol/g wet wt)	12.6 ± 0.5	12.1 ± 0.6	NS
Liver phospholipids (μmol/g wet wt)	36.0 ± 0.3	37.5 ± 0.7	NS
Liver cholesterol (μmol/g wet wt)	6.28 ± 0.17	5.61 ± 0.17	<0.001

[a] Data are expressed as mean ± SE; $n = 25$.
[b] P-values from one-way analysis of variance (ANOVA).

Table III. Influence of Dietary Copper on the Incorporation of ^{14}C-Acetate into Hepatic Total Fatty Acids[a]

Time (min)	Cu adequate	Cu deficient	ANOVA[b]
3	1928 ± 147	4155 ± 328	
6	3380 ± 157	7108 ± 506	Cu: <0.001
9	4893 ± 403	9420 ± 464	TL: < 0.001
12	6403 ± 440	12880 ± 1752	Cu × TL: <0.01

[a] Data are expressed as mean dpm/liver/100 g body wt ± SE; $n = 5$.
[b] P-value for analysis of variance (ANOVA) treatment comparisons; $df = 1$ for Cu, time linear (TL), time quadratic (TQ), time cubic (TC), and the following interaction: Cu × TL; Cu × TQ; Cu × TC.

Table IV. Influence of Diet on the Distribution of Radioactivity Among the Various Lipid Classes[a]

	Cu adequate	Cu deficient	ANOVA[b]
Triacylglycerols	48.20 ± 3.42	43.44 ± 1.86	NS
Phospholipids	27.73 ± 1.25	32.24 ± 6.51	NS
Cholesteryl esters	5.88 ± 1.26	3.74 ± 1.35	NS
Free fatty acids	14.03 ± 1.55	12.13 ± 3.39	NS
Unknown	4.29 ± 1.94	8.25 ± 2.12	NS

[a] Data are expressed as mean percentage of total radioactivity (at 9 min) ± SE; $n = 5$.
[b] P-value from one-way analysis of variance (ANOVA) ($P > 0.05$).

pholipids, and cholesteryl esters was 51%, 35%, and 8%, respectively.

A previous study was conducted by Banerjee and Redman (20) to determine the rate of incorporation of [2-^{3}H]glycerol into hepatic lipids in young chickens. Incorporation of the radioactive glycerol into hepatic lipids was linear until a peak was reached at 5 min and then dropped to 80% of the maximal level at 15 min after injection. In these young chickens, 27% of the total radioactivity associated with total hepatic lipids was incorporated into phospholipids. In the present study, the incorporation of [1-^{14}C]acetate into hepatic total fatty acids also increased linearly with time, reached the maximum at 12 min (Table III), and dropped to 74% of the peak level by 24 min (data not presented). The slight difference in response time between these studies may be due to the type of radioactive substrates and animal models used.

In an earlier study, the influence of Cu deficiency on the capacity of the liver to synthesize fatty acids from [1-^{14}C]acetate was determined by using liver slices (7). The capacity of liver slices, derived from Cu-deficient rats supplemented with no cholesterol, to synthesize fatty acids on a per gram basis was found to be 70% higher than that of adequate rats. When the data were expressed as nanomoles of acetate incorporated into fatty acids per liver organ corrected for body weight, a 2-fold increase in the capacity to synthesize fatty acids was demonstrated as a result of Cu deficiency. The present study established that hepatic total fatty acid synthesis (dpm/liver/100 g body wt) is increased about 2-fold *in vivo* in Cu-deficient rats (Table III). In addition, the proportion of nascent fatty acids incorporated into triacylglycerols, phospholipids, cholesteryl esters, and free fatty acids appeared not to be altered by Cu deficiency (Table IV).

The increase in hepatic lipogenesis may have resulted from increases in enzyme activities of glycolytic and fatty acid synthesis pathways and thus accelerated the flow of substrates through these pathways. Since hepatic triacylglycerol content is not affected by Cu deficiency (Table II), the fatty acids derived from the enhanced hepatic lipogenesis are most likely diverted to sustain an increase in lipoprotein production, leading to the observed hyperlipidemia. This enhanced lipogenesis may be in response to, or alternatively may have resulted in, the recently observed increase in the utilization of fat as a substrate for fuel in Cu-deficient rats (21). In Cu-deficient rats, the respiratory quotient value

Table V. Influence of Dietary Copper on the Incorporation of ^{14}C-Acetate into Fatty Acids of Hepatic Triacylglycerols and Phospholipids[a]

Time (min)	Cu adequate	Cu deficient	ANOVA[b]
Triacylglycerols (dpm/liver/100 g body wt)			
3	823 ± 102	1661 ± 205	
6	1574 ± 212	2998 ± 173	Cu: <0.001
9	2242 ± 122	4137 ± 477	TL: <0.001
12	3043 ± 331	6469 ± 642	Cu × TL:< 0.001
Phospholipids (dpm/liver/100 g body wt)			
3	544 ± 21	1256 ± 173	
6	952 ± 31	2288 ± 173	Cu: <0.001
9	1497 ± 81	3029 ± 375	TL: <0.001
12	1608 ± 91	4056 ± 408	Cu × TL: <0.01

[a] Data are expressed as mean ± SE; $n = 5$.
[b] *P*-value for analysis of variance (ANOVA) treatment comparison; $df = 1$ for Cu, time linear (TL), time quadratic (TQ), time cubic (TC), and the following interactions: Cu × TL; Cu × TQ; Cu × TC.

Table VI. Influence of Dietary Copper on the Incorporation of ^{14}C-Acetate into Fatty Acids of Hepatic Cholesteryl Esters and Free Fatty Acids[a]

Time (min)	Cu adequate	Cu deficient	ANOVA[b]
Cholesteryl esters (dpm/liver/100 g body wt)			
3	100.3 ± 3.6	121.0 ± 24.4	
6	225.3 ± 33.6	205.3 ± 3.8	
9	302.5 ± 29.0	346.4 ± 66.4	
12	372.1 ± 25.4	580.6 ± 90.0	TL: <0.001
Free fatty acids (dpm/liver/100 g body wt)			
3	294.4 ± 58.8	476.3 ± 107.0	
6	459.0 ± 33.7	767.8 ± 62.8	Cu: <0.01
9	685.6 ± 77.6	1158.0 ± 328.0	TL: <0.02
12	525.3 ± 75.7	826.5 ± 155.0	TQ: <0.03

[a] Data are expressed as mean ± SE; $n = 5$.
[b] *P*-value for analysis of variance (ANOVA) treatment comparison; $df = 1$ for Cu, time linear (TL), time quadratic (TQ), time cubic (TC), and the following interactions: Cu × TL; Cu × TQ; Cu × TC.

obtained from indirect calorimetry was reduced from 0.85 to 0.80, daily fat utilization was increased from 3.6 to 5.1 g/day, and carbohydrate usage was reduced from 7.9 to 5.2 g/day. This reduced preference of carbohydrate as a fuel substrate may indicate that dietary carbohydrate is not readily utilized by peripheral tissues and a substantial portion is converted into hepatic fatty acids before being used for fuel. This speculation is supported by the impaired glucose tolerance observed in Cu-deficient rats (22, 23). Thus, the enhanced hepatic fatty acid synthesis appears to be a mechanism to circumvent the reduced carbohydrate utilization observed in Cu-deficient rats.

In view of the markedly enlarged plasma volume associated with Cu deficiency, the pool sizes of all components in various lipoprotein fractions were markedly increased, with the exception of unaltered protein and phospholipid pool sizes and a small reduction in cholesterol pool size in VLDL (3). A disproportionately greater increase in triacylglycerol than cholesterol pool size was observed in all fractions, especially in VLDL and low density lipoproteins of Cu-deficient rats. This increase appeared not to be at the expense of liver triacylglycerol store because hepatic triacylglycerol content was unchanged in Cu-deficient rats (Table II). However, reductions in hepatic cholesterol content (24–26) and cholesterol percent composition of VLDL (3), observed in Cu-deficient rats fed no cholesterol supplements, suggest that cholesterol derived from the enhanced hepatic synthesis (27) and uptake of plasma high density lipoprotein cholesteryl ester (11) is unavailable or insufficient to maintain the normal cholesterol composition in VLDL and the liver. Thus, endogenous cholesterol synthesis may have reached its

maximum capacity, unable to sustain normal hepatic cholesterol concentration and lipoprotein cholesterol composition, and the limited supply of cholesterol may have blunted the overall increase in lipoprotein assembly and secretion. These findings, together with that of the present study, indicate that the new steady state of hyperlipoproteinemia associated with Cu deficiency is most likely induced by increases in lipogenesis and apolipoprotein synthesis rather than by cholesterol synthesis.

An overview of a new steady state in lipid and lipoprotein metabolism, as a result of Cu deficiency, can be deduced from the currently available data. First, cholesterol derived from an enhanced hepatic synthesis (27, 28) is packaged into lipoproteins to facilitate an accelerated clearance of newly synthesized cholesteryl ester from the liver to the plasma (17). The enhanced uptake of plasma high density lipoprotein cholesteryl esters by the liver indicated that an increased amount of plasma cholesterol may be recycled back to the liver (11). Since bile acid production and biliary cholesterol elimination are not affected by Cu deficiency (24), the increased supply of cholesterol from *de novo* synthesis (27, 28) and that recycled back from the plasma (11) is not diverted to the excretory route. Instead, all available hepatic cholesterol seemed to be used to sustain an accelerated secretion into the plasma, even to the extent of depressing the hepatic cholesterol content (29). Second, fatty acids derived from an enhanced hepatic lipogenesis (3, 7) are packaged into lipoproteins, resulting in a 6-fold increase in plasma VLDL triacylglycerol pool and 2-fold elevation in high density lipoprotein triacylglycerol pool. Third, the marked increase in hepatic apoA-I synthesis (30, 31) provided the necessary apolipoproteins for an enhanced lipoprotein synthesis and secretion. Finally, preliminary morphologic data of liver parenchymal cells revealed a marked increase in mitochondria number and in the network of rough endoplasmic reticulum, which are organelles required to sustain the enhanced lipoprotein synthetic processes (32). Thus, the hyperlipoproteinemia observed appears to be sustained by an increase in hepatic lipoprotein synthesis.

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