

# Low Dietary Zinc Alters Indices of Copper Function and Status in Postmenopausal Women

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**OBJECTIVES:** To better define the relationship between dietary zinc and copper for humans so that sound recommendations for intakes of these elements can be made.

**METHODS:** A study was conducted to ascertain the effect of moderately excessive and deficient intakes of zinc on copper metabolism and use in humans fed low and luxuriant amounts of copper. Twenty-one postmenopausal women housed in a metabolic unit completed the study as designed. After a 10-d equilibration period in which they were fed a diet providing 31.5  $\mu\text{mol}$  (2 mg) Cu and 91.8  $\mu\text{mol}$  (9 mg) Zn/8.4 MJ (2000 kcal), the women were divided into two groups. One group was fed a diet containing 15.7  $\mu\text{mol}$  (1 mg) Cu/8.4 MJ (2000 kcal), and the other group was fed a diet containing 47.2  $\mu\text{mol}$  (3 mg) Cu/8.4 MJ (2000 kcal). After equilibration, both groups were fed the basal diet providing 45.9  $\mu\text{mol}$  (3 mg) Zn/8.4 MJ (2000 kcal) for 90 d; this was followed by another 10-d equilibration period before dietary zinc was increased to 811  $\mu\text{mol}$  (53 mg)/8.4 MJ (2000 kcal) for 90 d.

**RESULTS:** The women were in positive copper balance only when the diet provided 47.2  $\mu\text{mol}$  (3 mg) Cu and 811  $\mu\text{mol}$  (53 mg) Zn/d. Immunoreactive ceruloplasmin concentrations and platelet cytochrome-c oxidase activity on a platelet number basis were significantly lower and the ratio between enzymatic and immunoreactive ceruloplasmin was significantly higher during low dietary than during high dietary zinc intake. Serum cholesterol was higher in subjects fed 15.7  $\mu\text{mol}$  (1 mg) Cu/d than in those fed 47.2  $\mu\text{mol}$  (3 mg) Cu/d. Total and low-density lipoprotein cholesterol concentrations decreased with zinc supplementation. Whole-blood glutathione concentration and erythrocyte glutathione peroxidase activity were lower during high than during low dietary zinc intake.

**CONCLUSIONS:** The findings indicate that an inadequate intake of zinc (45.9  $\mu\text{mol}/\text{d}$ ; 3 mg/d) was more effective than a moderately high intake of zinc (811  $\mu\text{mol}/\text{d}$ ; 53 mg/d) in inducing changes associated with a decreased copper status in postmenopausal women. Furthermore, the findings indicate that copper status indicators might be useful in evaluating changes in zinc status in humans, and an intake of 15.7  $\mu\text{mol}$  (1 mg)/d of copper may be inadequate for postmenopausal women. *Nutrition* 2001;17:701–708. ©Elsevier Science Inc. 2001

**KEY WORDS:** copper, ceruloplasmin, cytochrome c oxidase, superoxide dismutase, cholesterol, zinc, trace elements

## INTRODUCTION

In 1946, Smith and Larson<sup>1</sup> reported that extra dietary copper could partly prevent anemia induced in rats by very high dietary

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zinc. Since that report, numerous others have appeared showing that extremely high amounts of zinc interfere with the uptake and metabolism of copper. For example, Van Campen<sup>2</sup> reported that zinc:copper ratios of 150 to 600 resulted in decreased absorption of a <sup>64</sup>Cu dose in rats. Hill and Matrone<sup>3</sup> found that 3.44 to 4.97 mmol (225 to 325 mg) of Zn/kg of diet decreased hemoglobin and weight and increased mortality in chicks, which they attributed to induced copper deficiency. More recently, zinc supplementation, e.g., 2.3 mmol (150 mg)/d for an adult, has been used as a therapeutic agent for the treatment of Wilson's disease, an inherited, autosomal recessive disease of copper accumulation.<sup>4</sup> Thus, there is no question that extremely high amounts of zinc are antagonistic to copper metabolism.

The findings with extremely high dietary zinc has resulted in the dogma that moderately high intakes of zinc, even those close to the recommended dietary allowance, should be considered a toxic risk because of the possibility of inducing copper-deficiency pathology in humans. Sandstead<sup>5</sup> reviewed the evidence that has resulted in this dogma. However, although the evidence seems compelling, there are some reported discrepancies and findings that challenge the unequivocal acceptance of the dogma. The signs of copper deprivation supposedly induced by moderately high intakes of zinc include decreased high-density lipoprotein (HDL) cholesterol<sup>6</sup> and erythrocyte superoxide dismutase (ESOD).<sup>7,8</sup>

However, decreased HDL-cholesterol is not specific to copper deprivation and ESOD can be altered by factors other than copper deprivation. Interestingly, more specific copper-deprivation signs such as decreased serum ceruloplasmin and platelet cytochrome-c oxidase have not been reported to be induced by moderate zinc supplementation. Moreover, decreased HDL-cholesterol has not been consistently found with moderate zinc supplementation.<sup>9,10</sup> Pachotikarn et al.<sup>9</sup> concluded that, because 765  $\mu\text{mol}$  (50 mg) of Zn/d for 6 wk tended to decrease plasma cholesterol and increase HDL-cholesterol in males fed a marginal copper intake, humans might differ from animals (rats) in the cholesterol response when a high ratio of zinc to copper (60:1) is fed. Freeland-Graves et al.<sup>10</sup> fed zinc supplements to women to achieve zinc:copper ratios ranging from 3 to 64 for 6 wk. They observed no uniform or sustained response of plasma total or HDL-cholesterol as the ratios were increased; actually, there was more of a trend toward hypocholesterolemia than hypercholesterolemia with increased ratios.

The finding that moderate amounts of zinc decreases ESOD also might not be directly related to a change in copper status. Antioxidant activity involving zinc can be accomplished through mechanisms other than ESOD. For example, zinc supplementation can increase metallothionein in erythrocytes<sup>11</sup>; metallothionein has been shown to have an antioxidant function.<sup>12</sup> Thus, the finding that zinc supplementation increases erythrocyte metallothionein and decreases ESOD suggests an inverse relationship between these two antioxidants not necessarily caused by changed copper status.

It is surprising that there has been essentially no examination of the possibility that moderate zinc deficiency increases the need for copper and thus could induce copper deficiency changes in people fed marginal amounts of copper. If zinc cannot fully perform its antioxidant functions, the need for copper, which has antioxidant functions, may be increased.

The preceding discussion shows that there is a need to better define the relationship between dietary zinc and copper in humans so that sound recommendations regarding relative intakes of these elements can be made. Thus, we conducted a study to determine the effect of moderately deficient and moderately excessive intakes of zinc on copper metabolism and use in humans fed marginal and adequate amounts of copper. The marginal amount of copper (about 15.7  $\mu\text{mol}/8.4$  MJ; 1 mg/2000 kcal) was considered so because it was less than the lower limit of the Estimated Safe And Adequate Daily Dietary Intake (ESADDI) established by the Food and Nutrition Board.<sup>13</sup> The adequate intake (47.2  $\mu\text{mol}$  or 3 mg/d) was the upper limit of the ESADDI; the ESADDI for copper is 23.6 to 47.2  $\mu\text{mol}$  (1.5 to 3.0 mg)/d. The upper limit was used to ensure the prevention of the potential induction of copper deficiency by the high dietary zinc. The zinc:copper ratios ranged from 1 to 53 in the experimental diets.

## SUBJECTS AND METHODS

Twenty-eight postmenopausal women were recruited for the study after they had been informed in detail both verbally and in writing of the nature of the research and associated risks, and after medical, psychological, and nutritional evaluations had established that they had no underlying disease and were emotionally suited for the project. One volunteer was dismissed shortly after the study started because of high blood pressure that was not present during recruitment; another volunteer was dismissed because of diet incompatibility; and one volunteer left the study for personal reasons. Protocols were approved by the Institutional Review boards of the University of North Dakota and the US Department of Agriculture and followed the Guidelines of the Department of Health and Human Services and the Helsinki Declaration regarding the use of human subjects.

The study was conducted at two different times, with about half of the women participating each time. The 25 women who com-

pleted the study were between the ages of 50 and 76 y ( $64.9 \pm 6.7$  y, mean  $\pm$  standard deviation) at entry. They were  $159.6 \pm 7.6$  cm tall and weighed  $65.1 \pm 9.5$  kg at the beginning of the study.

The women were maintained in a metabolic unit under close supervision for 200 d. The environment and subject management were as described previously.<sup>14</sup> They were fed a constant weighed diet of conventional foods that was low in copper (9.6  $\mu\text{mol}/8.4$  MJ; 0.6 mg/2000 kcal) and zinc (45.9  $\mu\text{mol}/8.4$  MJ; 3 mg/2000 kcal) on a 3-d menu rotation. Details of the diet have been described.<sup>15</sup> The diet was adequate in all other known nutrients. During an initial 10-d equilibration period, the subjects were fed the basal diet supplemented with 22  $\mu\text{mol}$  (1.4 mg) of Cu per day (31.5  $\mu\text{mol}$  total or 2 mg) and 91.8  $\mu\text{mol}$  (6 mg) of Zn per day (137.7  $\mu\text{mol}$  or 9 mg total). After equilibration and initial testing, the women were assigned to two groups: one group was fed the basal diet supplemented with 6.3  $\mu\text{mol}$  (0.4 mg) of Cu/2000 (8.4 MJ) kcal (total of 15.7  $\mu\text{mol}/8.4$  MJ; 1 mg/2000 kcal) and the other group was fed the basal diet supplemented with 37.8  $\mu\text{mol}$  (2.4 mg) of Cu/d (a total of about 47.2  $\mu\text{mol}/8.4$  MJ; 3.0 mg/2000 kcal). The remaining 190 d had two 90-d dietary periods for both groups. The basal diet (45.9  $\mu\text{mol}$  Zn/8.4 MJ; 3.0 mg/2000 kcal) with no zinc supplement was fed for the first 90-d period, and the diet supplemented with 765  $\mu\text{mol}$  (50 mg) Zn/d was fed for the second 90-d period. The two 90-d periods were separated by a second equilibration period of 10 d, during which the basal diet was supplemented with 22  $\mu\text{mol}$  (1.4 mg) of Cu and 91.8  $\mu\text{mol}$  (6 mg) of Zn per day. Zinc was supplemented as zinc gluconate and copper was supplemented as cupric sulfate in beverages served with the meals. All other aspects of the diet remained constant throughout the study.

Foods were weighed to an accuracy of 1% during preparation in the metabolic kitchen and eaten quantitatively by the women. The dietary intake of each woman was based on energy needs as calculated by the Harris-Benedict equation,<sup>16</sup> plus an additional 60% of basal energy expenditure for normal activity. So that individual body weights were maintained to within 2% of admission weights, the amount of basal diet fed was adjusted as necessary in 0.84-MJ (200-kcal) increments by proportionally changing the amounts of all foods.

Urine and feces were collected continuously during the last 78 d of each 90-d dietary period with precautions to avoid trace-mineral contamination. Duplicate diets at 8.4 MJ (2000 kcal) were prepared daily for analysis and blended in a plastic blender with stainless-steel blades. Adjustments for differences in individual energy intakes were calculated proportionally.

Copper and zinc were determined in 6-d composites for diets and feces and measured by inductively coupled argon plasma emission spectroscopy (Perkin-Elmer, Norwalk, CT, USA) after wet digestion of aliquots of freeze-dried material with nitric and perchloric acids.<sup>17</sup> Urinary copper and zinc were determined by analysis with inductively coupled argon plasma emission spectroscopy of a diluted aliquot. Concurrent replicate analysis of standard bovine liver SRM 11577b ( $n = 28$ ; National Institute of Standards and Technology, Gaithersburg, MD, USA) yielded values of  $156 \pm 6$  and  $118 \pm 8$   $\mu\text{g}/\text{g}$  as compared with certified values of  $160 \pm 8$  and  $127 \pm 16$   $\mu\text{g}/\text{g}$  for copper and zinc, respectively. Replicate analysis of Total Diet SRM 1548 ( $n = 4$ ) yielded values of  $2.6 \pm 0.05$  and  $24.6 \pm 4.8$   $\mu\text{g}/\text{g}$  as compared with certified values of  $2.6 \pm 0.3$  and  $30.8 \pm 1.1$   $\mu\text{g}/\text{g}$  for copper and zinc, respectively, and concurrent analysis of a diet pool ( $n = 11$ ) yielded values of  $0.52 \pm 0.07$  and  $2.1 \pm 0.2$   $\mu\text{g}/\text{g}$  as compared with expected ranges of 0.43 to 0.53 and 1.7 to 2.5  $\mu\text{g}/\text{g}$  for copper and zinc, respectively. Replicate concurrent analysis of a fecal pool ( $n = 26$ ) yielded values of  $3.6 \pm 0.2$  and  $19.6 \pm 1.4$   $\mu\text{g}/\text{g}$  as compared with expected ranges of 3.0 to 4.6 and 16.9 to 24.5  $\mu\text{g}/\text{g}$  for copper and zinc, respectively.

Because potentially harmful electrocardiographic changes have been found in people depleted in copper, Holter electrocardiograms were done frequently (twice during initial equilibration and

then during weeks 4, 7, 9, 10, 11, and 12 of each 90-d dietary period) on all subjects. Tapes obtained were scanned (model 363, Accuplus, Del Mar Avionics, Irvine, CA, USA) by skilled nurses. Ventricular premature discharges were identified and counted. If a verified four-fold increase in the baseline value obtained during equilibration occurred in the volunteers on the low-copper diet, they were given the 37.8  $\mu\text{mol}$  (2.4 mg)/d copper supplement until the end of the study.

Blood was drawn at weekly intervals into plastic syringes from antecubital veins that had been distended by temporary use of a tourniquet after the subjects had fasted for 12 h. An average of no more than 235 mL/mo was drawn. Aliquots were mixed with appropriate anticoagulants and processed within 90 min of the time the blood was drawn. Plasma zinc and copper concentrations were determined by flame atomic absorption spectrometry after dilution with deionized water.<sup>17</sup> Ceruloplasmin (EC 1.16.3.1) was determined enzymatically<sup>18</sup> and immunochemically (BN 1000 Nephelometer, Behring Diagnostics, Inc., Westwood, MA, USA). We normalized the results of both assays to milligrams of ceruloplasmin per liter by using purified human ceruloplasmin (40 U/mg of protein; Sigma, St. Louis, MO, USA) as a standard. Erythrocyte Cu,Zn superoxide dismutase (EC 1.15.1.1) activity was measured by inhibition of pyrogallol autooxidation.<sup>19</sup> Platelet cytochrome-c oxidase (EC 1.9.3.1) was determined by using previously described methods.<sup>20,21</sup>

A Coulter S+IV hematology analyzer (Coulter Electronics, Inc., Hialeah, FL, USA) was used to determine hematologic indices. Serum cholesterol, HDL-cholesterol, and low-density lipoprotein (LDL) cholesterol concentrations were determined by using a Cobas Fara II Centrifugal analyzer (Roche Diagnostics Systems, Montclair, NJ, USA). Glutathione<sup>22</sup> and glutathione peroxidase activity<sup>23</sup> were determined by previously described methods.

Changes in some of the biochemical variables appeared only toward the end of each 90-d dietary period. Thus, to ensure that changes were recognized, only the last two measurements of each of the variables were used in the statistical analysis. The last three 6-d balance periods were statistically analyzed because they encompassed the blood draws for the statistically analyzed biochemical variables. The data were analyzed by two-way repeated measures analysis of variance (diet zinc and copper) with a SAS general linear model program (SAS version 6.12, SAS Institute, Cary, NC, USA). Tukey's contrasts differentiated among means for variables that had been significantly affected by the treatments.  $P \leq 0.05$  was considered significant. Variances in the data were expressed as a pooled standard deviation, calculated as the square root of the mean square error from the analysis of variance.

## RESULTS

Three of the women fed the diet marginal in copper (15.7  $\mu\text{mol}/\text{d}$ ; 1 mg/d) exhibited an increase in ventricular premature discharges, resulting in obligatory supplementation with copper before the end of the study. This occurred in two of the subjects during the low-zinc dietary period and shortly before the scheduled end of the study in one subject during the high-zinc dietary period. The two subjects on low dietary zinc continued to exhibit increased, but fluctuating, numbers of abnormal discharges with copper supplementation. After the switch to high dietary zinc, the return to control numbers occurred. None of the women receiving 47.2  $\mu\text{mol}$  (3 mg) of Cu/d had significant changes in their electrocardiograms. Data from the two subjects who were in the low dietary copper groups and then supplemented with copper midway in the study because of heart-rhythm changes are not included in the reported results. After the study, we discovered that two women were using an adhesive containing extremely high amounts of zinc for their false teeth; their data also are not included in the reported values.

Table I shows that a significant interaction between zinc and

TABLE I.

EFFECT OF ZINC AND COPPER INTAKES ON COPPER BALANCE ( $\mu\text{MOL}/\text{D}$ ) DURING THE LAST 18 D OF EACH 90-D DIETARY PERIOD					
<i>n</i> *	Diet Zn	Diet Cu	Fecal Cu	Urine Cu	Cu balance
9	46.2	16.2	16.4	1.0	-1.4
9	811	16.4	16.8	1.0	-1.6
12	48.3	47.8	47.8	0.9	-0.8
12	817	48.0	44.0	1.0	2.8
Pooled SD			2.7	0.3	3.0
<i>P</i> †					
Zn effect			0.06	0.58	0.07
Cu effect			0.0001	0.91	0.07
Zn $\times$ Cu			0.02	0.58	0.05

\* Number of subjects.

† Analysis of variance.

Cu, copper; SD, standard deviation; Zn, zinc.

copper affected copper balance during the last 18 d of each 90-d dietary zinc period. This significant interaction occurred because only the women fed high dietary zinc and luxuriant copper had a positive copper balance (2.83  $\mu\text{mol}/\text{d}$ ; 0.18 mg/d); copper balance was not positive in all other groups. High dietary zinc did not exacerbate the non-positive copper balance in the women fed low dietary copper. Moreover, 47.2  $\mu\text{mol}$  (3 mg) of Cu/d did not induce positive copper balance in the women fed low dietary zinc. The difference in copper balance with high dietary copper and zinc apparently was the result of a lower amount of dietary copper lost in the feces. Urinary copper was not affected by dietary treatment.

Table II shows that zinc balance reflected dietary zinc intake and was not significantly affected by copper intake. The percentage of dietary zinc intake excreted in the feces and the daily excretion in the urine were increased when dietary zinc was increased. Zinc balance was near zero but did not become non-positive during low zinc intake, however, the balance determinations did not include phlebotomy and surface losses of zinc.

Copper-status indicators were variably affected by dietary treatment (Tables III and IV). In Table III the initial equilibration data are presented to show values that would reflect more normal zinc and copper intakes than during the periods of low and high dietary zinc intake. Thus, the percentage change from initial equilibration-period values shown in Table IV were used to gain insight into whether induced changes by low or high dietary zinc or copper resulted in more or less normal values. Plasma copper concentrations tended to be lower in the women fed 15.7  $\mu\text{mol}$  (1 mg) of Cu/d than in those fed 47.2  $\mu\text{mol}$  (3 mg) of Cu/d ( $P < 0.07$ ), regardless of dietary zinc. This decrease in plasma copper was significant ( $P < 0.02$ ) when expressed as a percentage change from equilibration concentrations to concentrations during low and during high dietary copper intake (Table IV). Zinc intake did not significantly affect plasma copper. Serum enzymatic ceruloplasmin was not significantly affected by dietary treatment, but dietary zinc affected serum-immunoreactive ceruloplasmin and the specific activity of ceruloplasmin defined as the ratio between enzymatic and immunoreactive ceruloplasmin (ENZ/IMM Cp). Immunoreactive ceruloplasmin concentrations were significantly lower ( $P < 0.05$ ) and ENZ/IMM Cp ratios significantly higher during low dietary zinc intake than during high dietary zinc intake. High zinc with 47.2  $\mu\text{mol}$  (3 mg) of Cu/d copper was the dietary treatment that resulted in values closest to those obtained during the initial equilibration for immunoreactive ceruloplasmin and ENZ/IMM Cp.

Platelet cytochrome-c oxidase (U/10<sup>9</sup>) and ESOD also were

TABLE II.

EFFECT OF ZINC AND COPPER INTAKES ON ZINC BALANCE DURING THE LAST 18 D OF EACH 90-D DIETARY PERIOD							
<i>n</i> *	Dietary Zn ( $\mu\text{mol/d}$ )	Dietary Cu ( $\mu\text{mol/d}$ )	Fecal Zn ( $\mu\text{mol/d}$ )	Fecal Zn (% intake)	Urine Zn ( $\mu\text{mol/d}$ )	Urine Zn (% intake)	Zn balance ( $\mu\text{mol/d}$ )
9	46.2	16.2	36.7	79.5	4.4	9.55	5.4
9	811	16.4	737	90.8	18.6	2.31	56.0
12	48.3	47.8	37.1	77.3	3.8	7.62	7.3
12	817	48.0	673	82.4	16.2	1.99	127.4
Pooled SD			49	8.8	3.5	2.8	49.1
<i>P</i> †							
Zn effect			0.002	0.007	0.02	0.0001	0.10
Cu effect			0.20	0.38	0.53	0.20	0.11
Zn $\times$ Cu			0.52	0.28	0.51	0.36	0.49

\* Number of subjects.

† Analysis of variance.

Cu, copper; SD, standard deviation; Zn, zinc.

significantly affected by dietary zinc. ESOD activity was significantly lower ( $P < 0.02$ ) at the end of the zinc-supplementation period than at the end of the low-zinc dietary period (Table III). ESOD activity decreased significantly during the low-zinc dietary period in the women fed low dietary copper (Table IV). Conversely, ESOD activity increased during the low dietary zinc period in the women fed 47.2  $\mu\text{mol}$  (3 mg) of Cu/d. As shown in Table IV, ESOD activity decreased in both groups when dietary zinc was changed from 45.9  $\mu\text{mol}$  (3 mg) to 811  $\mu\text{mol}$  (53 mg)/d. Platelet cytochrome-c oxidase activity, on a platelet-number basis, increased significantly when supplemental zinc was fed ( $P < 0.0007$ ). Dietary copper had no significant effect on this measure. When expressed as milligrams of protein, neither zinc nor copper significantly affected cytochrome-c oxidase activity. This apparent discrepancy was the result of a reduction in platelet protein during the low-zinc dietary period followed by an increase in platelet protein when the zinc supplement was fed (Table III).

Total cholesterol was higher at the end of both dietary periods

in the women fed 15.7  $\mu\text{mol}$  (1 mg) of Cu/d than in those receiving 47.2  $\mu\text{mol}$  (3 mg) of Cu/d ( $P < 0.05$ ; Table V). The significance of this cross-sectional finding was made questionable by the fact that the women fed the low-copper diet had higher cholesterol during the initial equilibration than did the women fed 47.2  $\mu\text{mol}$  (3 mg) of Cu/d. Table VI shows that dietary zinc had a significant effect on total cholesterol. During the low-zinc dietary period, total cholesterol increased over the initial equilibration value; there was a tendency for a greater increase in cholesterol in the women consuming the low-copper diet. Increasing the zinc intake to 811  $\mu\text{mol}$  (53 mg)/d lowered serum cholesterol concentrations. LDL-cholesterol changes were similar to the total-cholesterol changes. HDL-cholesterol (Tables V and VI), very LDL-cholesterol, and triacylglycerols (data not shown) were not significantly affected by the dietary manipulations.

Whole-blood glutathione and erythrocyte glutathione peroxidase activity were significantly affected by the dietary zinc but not by dietary copper. Glutathione concentrations were increased from

TABLE III.

EFFECT OF ZINC AND COPPER INTAKE ON COPPER INDICATORS*									
Diet	<i>n</i>	Serum ceruloplasmin				Erythrocyte superoxide dismutase (U/mg protein)	Platelet		
		Plasma Cu ( $\mu\text{mol/L}$ )	ENZ (mg/L)	IMM (mg/L)	ENZ/IMM		Cytochrome-c oxidase		Protein (mg/mL)
						U/10 <sup>9</sup>	U/mg protein		
Initial equilibration	9	17.79	467	364	1.29	69.8	3.18	1.33	2.53
Low Cu, low Zn	9	15.90	426	311	1.36	60.3	3.37	1.61	2.10
Low Cu, high Zn	9	15.49	399	324	1.25	57.3	4.29	1.65	2.65
Initial equilibration	12	18.57	480	377	1.28	55.9	3.34	1.40	2.46
High Cu, low Zn	12	17.79	468	337	1.39	65.6	3.22	1.78	1.83
High Cu, high Zn	12	17.88	451	363	1.24	60.8	3.82	1.64	2.34
Pooled SD		1.01	31	29	0.10	5.5	0.61	0.28	0.19
<i>P</i> †									
Zn effect		0.94	0.42	0.05	0.0003	0.03	0.0007	0.61	0.07
Cu effect		0.07	0.17	0.12	0.64	0.49	0.55	0.85	0.05
Zn $\times$ Cu		0.79	0.94	0.47	0.57	0.61	0.40	0.31	0.48

\* Values are the means of the last two blood draws.

† Analysis of variance.

Cu, copper; ENZ, enzymatic; IMM, immunoreactive; SD, standard deviation; Zn, zinc.

TABLE IV.

	PERCENTAGE CHANGE FROM PREVIOUS DIET PERIOD—COPPER INDICATORS							
	Plasma Cu		Erythrocyte superoxide dismutase		Serum ceruloplasmin			
	Low Cu	High Cu	Low Cu	High Cu	IMM		ENZ/IMM	
					Low Cu	High Cu	Low Cu	High Cu
Equilibration to low Zn	-10.2	-4.3	-14.7	17.2	-12.6	-10.0	6.3	8.9
Low Zn to high Zn	-2.8	0.7	-3.9	-6.1	3.3	8.5	-8.1	-10.3
<i>P</i> *								
Zn effect		0.35		0.15		0.002		0.0008
Cu effect		0.03		0.0001		0.54		0.75
Zn × Cu		0.69		0.0006		0.78		0.59

\* Analysis of variance.

Cu, copper; ENZ, enzymatic; IMM, immunoreactive; Zn, zinc.

equilibration during the low-zinc dietary period and returned to essentially equilibration concentrations during the high-zinc dietary period (Table V). Erythrocyte glutathione peroxidase activity also was increased by low dietary zinc and decreased by high dietary zinc. However, the decrease did not result in a return to initial equilibration activity; the women fed 47.2  $\mu\text{mol}$  (3 mg) of Cu/d and high dietary zinc came the closest to returning to initial equilibration activity. Table V also shows that low dietary zinc did not decrease plasma zinc concentrations below those present during initial equilibration. High dietary zinc significantly increased plasma zinc concentrations.

Surprisingly, the concentration of zinc in red blood cells was not affected by dietary zinc, nor was the concentration of copper affected by dietary copper (Table VII). However, high dietary zinc significantly decreased the concentration of copper in red blood cells, expressed as per gram of hemoglobin or per liter of packed cells.

Most of the measured indicators of iron status were unaffected

by the dietary treatments and thus are not presented. These include serum iron, hematocrit, and percentage of transferrin saturation. Hemoglobin was lower at the end of the high-zinc dietary period than the low-zinc dietary period in subjects with low and high dietary intakes of copper (131 versus 126 g/L,  $P < 0.001$ ). Most of the apparent drop in hemoglobin occurred during the last month of the zinc-supplementation period, probably because of accumulated blood loss from phlebotomy; the women maintained their hemoglobin concentrations during the low-zinc dietary period.

## DISCUSSION

The most surprising finding in this study was that an inadequate intake of zinc (45.9  $\mu\text{mol}/\text{d}$ ; 3 mg/d), not the moderately high intake of zinc (811  $\mu\text{mol}/\text{d}$ ; 53 mg/d), was the dietary treatment that induced changes associated with decreased copper status in postmenopausal women. This is exemplified by the copper-balance

TABLE V.

EFFECT OF ZINC AND COPPER ON SERUM TOTAL, HDL-, AND LDL-CHOLESTEROL, WHOLE-BLOOD GLUTATHIONE, ERYTHROCYTE GLUTATHIONE PEROXIDASE, AND PLASMA ZINC*							
	<i>n</i>	Serum cholesterol			Whole-blood glutathione (mmol/L)	Erythrocyte glutathione peroxidase (U $\dagger$ /g hemoglobin)	Plasma Zn ( $\mu\text{mol}/\text{L}$ )
		Total (mmol/L)	HDL (mmol/L)	LDL (mmol/L)			
Initial equilibration	9	5.59	1.29	3.67	1.004	2.04	12.7
Low Cu, low Zn	9	6.21	1.18	4.37	1.033	3.48	13.0
Low Cu, high Zn	9	6.00	1.20	4.09	0.949	2.95	17.1
Initial equilibration	12	5.04	1.32	3.13	1.095	2.52	13.2
High Cu, low Zn	12	5.38	1.23	3.54	1.134	3.33	13.8
High Cu, high Zn	12	5.17	1.15	3.47	1.001	2.97	16.4
Pooled SD		0.37	0.07	0.37	0.088	0.38	1.8
<i>P</i> ‡							
Zn effect		0.10	0.70	0.13	0.0009	0.002	0.0001
Cu effect		0.02	0.93	0.03	0.14	0.79	0.88
Zn × Cu		0.95	0.51	0.38	0.36	0.48	0.22

\* Values are the means of the last two blood draws.

† Units are moles of glutathione oxidized per minute.

‡ Analysis of variance.

Cu, copper; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation; Zn, zinc.

TABLE VI.

PERCENTAGE CHANGE FROM THE PREVIOUS DIET PERIOD— LIPIDS	<i>P</i> *				
	Equilibration to low Zn	Low Zn to high Zn	Zn × Cu		
			Zn effect	Cu effect	Zn × Cu
Total cholesterol					
Low Cu	11.9	−3.0	0.005	0.26	0.60
High Cu	7.4	−3.2			
LDL-cholesterol					
Low Cu	20.7	−5.6	0.003	0.74	0.45
High Cu	15.3	−1.3			
HDL-cholesterol					
Low Cu	−8.2	1.2	0.46	0.22	0.67
High Cu	−8.0	−4.9			
Glutathione					
Low Cu	4.7	−8.0	0.004	0.83	0.64
High Cu	5.2	−11.8			
Glutathione peroxidase					
Low Cu	94.6	−12.9	0.13	0.64	0.75
High Cu	64.2	−6.8			

\* Analysis of variance.

Cu, copper; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Zn, zinc.

data. When high dietary zinc was fed, the non-positive copper balance and high fecal excretion of copper were not exacerbated in the women fed low dietary copper. When copper intake was adequate, 811  $\mu\text{mol}$  (53 mg) of Zn/d did not induce a non-positive copper balance like low dietary zinc. This finding suggests that low dietary zinc can lead to low-copper status. The positive copper balance with high dietary zinc most likely was not an undesirable finding; it probably reflected a repletion of copper stores that were reduced by 90 d of non-positive balance during the period of low

TABLE VII.

EFFECT OF ZINC AND COPPER INTAKES ON COPPER AND ZINC CONTENT OF RED BLOOD CELLS*					
	<i>n</i>	Cu ( $\mu\text{mol}$ )		Zn ( $\mu\text{mol}$ )	
		g hemoglobin	L packed cells	g hemoglobin	L packed cells
Initial equilibration	9	0.039	13.0	0.52	173
Low Cu, low Zn	9	0.038	12.6	0.53	178
Low Cu, high Zn	9	0.032	10.6	0.53	173
Initial equilibration	11	0.041	13.9	0.50	166
High Cu, low Zn	12	0.036	12.2	0.52	174
High Cu, high Zn	12	0.033	10.8	0.51	170
Pooled SD		0.003	1.0	0.036	12
<i>P</i> †					
Zn effect		0.0008	0.0001	0.83	0.19
Cu effect		0.84	0.84	0.94	0.95
Zn × Cu		0.37	0.30	0.92	0.94

\* Values are means of the last two draws.

† Analysis of variance.

Cu, copper; SD, standard deviation; Zn, zinc.

dietary zinc. It is unclear whether the difference in copper balance was the result of impaired absorption or lower endogenous excretion of copper. Nonetheless, the balance findings were unexpected because human<sup>5</sup> and animal<sup>2</sup> studies have shown that copper absorption and balance are impaired when very high amounts of dietary zinc are fed. Conversely, the present results are consistent with those of Turmlund et al.<sup>24</sup> who showed that copper absorption in young men was higher (48% versus 38%) when 252  $\mu\text{mol}$  (16.5 mg)/d rather than when 84  $\mu\text{mol}$  (5.5 mg)/d of zinc was fed.

The immunoreactive-ceruloplasmin findings also indicate that a moderately deficient intake of zinc is more detrimental to copper metabolism and function than a moderately high intake. With this indicator of copper status, the value obtained indicative of decreased copper status was most marked when dietary copper and zinc were low. High dietary zinc seemed to improve this indicator of copper status; indeed, the value obtained when dietary copper and zinc were supplemented was similar to the initial equilibration value. The changes in the specific activity of ceruloplasmin (ENZ/IMM Cp) were the result of the significant drop in the immunoreactive-ceruloplasmin protein during the phase of low dietary zinc in the study, followed by a partial recovery when zinc supplements were fed. Yadrick et al.<sup>8</sup> also found a small but non-significant increase in immunoreactive-ceruloplasmin protein when zinc supplements of 756  $\mu\text{mol}$  (50 mg)/d were fed to young women. Because zinc can affect protein formation at transcription, translation, and posttranslation levels, zinc status affecting the formation of ceruloplasmin rather than copper status might have influenced the ENZ/IMM Cp findings.

Another suggested indicator of low-copper status is decreased platelet cytochrome-c oxidase activity.<sup>25</sup> Zinc supplementation did not change this variable in the direction that would indicate decreased copper status in the present study. When examined on the basis of units per  $10^9$  cells, instead of decreasing cytochrome-c oxidase activity, a zinc supplement of 756  $\mu\text{mol}$  (50 mg)/d increased the activity in the subjects fed low dietary copper. It was zinc deprivation that resulted in changes in cytochrome-c oxidase activity that might be construed as indicative of decreased-copper status. In contrast to the preceding, because the dietary manipulations apparently affected platelet protein content, cytochrome-c oxidase activity expressed as units per milligram of protein was not significantly affected by dietary zinc or copper. The findings of cytochrome-c oxidase and platelet protein suggest that, like ceruloplasmin, low and moderately high dietary zinc affect copper-associated variables through factors other than a direct effect on copper status.

Regardless of dietary copper, going from low dietary zinc to high dietary zinc decreased ESOD. Interestingly, low dietary zinc markedly increased ESOD in women fed adequate copper. However, the largest drop in ESOD activity from equilibration (−14.9%) occurred in the women fed low dietary zinc and copper. These findings suggest that factors other than zinc altering copper metabolism were affecting ESOD activity. In other words, perhaps changes in reactive oxygen metabolism were more a factor in the changes in ESOD activity than a direct effect of copper and zinc status on ESOD formation and activation. It is unfortunate that blood metallothionein measurements were not done. We arranged to have those measurements done in another laboratory, but those values were highly suspect and variable. Discussions with the leader of the other laboratory convinced us that those values probably were not valid and we did not consider them. Another study is needed to ascertain whether the antioxidant action of zinc via metallothionein varies inversely with ESOD. Nonetheless, the ESOD findings suggest that the decreases in ESOD activity seen in other studies<sup>7,8</sup> were not caused by an interference with copper metabolism.

The findings concerning glutathione and glutathione peroxidase support the suggestion that the supplemental zinc was affecting ESOD through mechanisms other than inducing decreased-copper status, with one possible mechanism being the changing of relative

amounts of antioxidants. Copper deficiency in rats increases plasma total glutathione, which is attributable to increases in reduced glutathione.<sup>26</sup> In the present study, whole-blood glutathione concentration was increased by low dietary zinc, not by supplemental zinc, which suggests that low dietary zinc induced a change similar to that of low dietary copper. However, tissue glutathione peroxidase is decreased in copper-deficient animals.<sup>26</sup> Low dietary zinc increased erythrocyte glutathione peroxidase activity in this study, which seems to contradict the suggestion that low dietary zinc has an effect similar to copper deficiency on variables responsive to dietary copper. However, the high values of glutathione peroxidase obtained during the low dietary zinc period were most different from the initial equilibration values. Thus, perhaps ESOD and glutathione peroxidase levels were decreased during zinc supplementation because of a reduced need for these antioxidants when circulating zinc was increased (but still in the normal range), as it was in the present study.

The cholesterol findings show that low dietary zinc can influence changes caused by a decrease in copper status. An increase in serum cholesterol is a consistent consequence of copper deficiency in growing animals and occasionally humans depleted of copper.<sup>27</sup> In this study, the combination of low dietary zinc with marginally low dietary copper for 13 wk produced the most marked increase in serum cholesterol over the equilibration value. Supplementing high zinc after the low-zinc period reduced serum cholesterol even in the women fed low copper for 26 wk; it also significantly lowered the concentrations of LDL-cholesterol. The effect of a milder zinc deprivation on cholesterol in this study is in contrast to the finding of decreased serum cholesterol in women fed a formula diet containing negligible amounts of zinc (216  $\mu\text{mol/d}$ ; 0.17 mg/d) for 35 d.<sup>28</sup>

The cholesterol findings do not support the hypothesis that an elevated dietary zinc:copper ratio will cause hypercholesterolemia in humans. This hypothesis was initially based on rat studies in which a zinc:copper ratio greater than 17.0 (including drinking water and basal diet sources of zinc and copper) resulted in the development of hypercholesterolemia.<sup>29</sup> Other studies with adult human subjects using zinc:copper ratios similar to those in the present study also have not supported this hypothesis.<sup>9,10</sup> Black et al.<sup>30</sup> also found that serum total cholesterol, LDL-cholesterol, and very LDL-cholesterol were not significantly affected in a 12-wk double-blind study in which men were fed a placebo or 0.765 mmol (50 mg) or 1.148 mmol (75 mg)/d supplements of zinc. However, they noted that HDL-cholesterol decreased in subjects fed the 1.148 mmol (75 mg)/d zinc supplement at 6 and 12 wk and those fed the 0.765 mmol (50 mg)/d zinc supplement at 12 wk. Goodwin et al.<sup>6</sup> reported that small (8.05 mmol/L) but significant increases (4%) in HDL-cholesterol occurred in 22 physically active subjects when they stopped taking zinc supplements, providing a median of 367  $\mu\text{mol}$  (24 mg)/d for 8 wk. HDL-cholesterol measurements were done only at the beginning and end of this study on subjects whose diets were not experimentally controlled. In the present study, no dietary manipulation, including 811  $\mu\text{mol}$  (53 mg) of Zn/d for 13 wk, affected HDL-cholesterol. However, much higher zinc supplementation usually does have an impact on HDL-cholesterol. Young women displayed a transient decrease in HDL-cholesterol at 4 wk when fed 1.53 mmol (100 mg) of Zn/d; but no significant change was seen at 8 wk.<sup>31</sup> In normal men, intakes of 2.45 mmol (160 mg) Zn/d for 5 wk significantly decreased HDL-cholesterol.<sup>32</sup> Even higher doses of zinc (300 mg/d; 4.59 mmol/d) raised LDL-cholesterol and decreased HDL-cholesterol.<sup>33</sup> Thus, it apparently is not so much the zinc:copper ratio that determines the effect of zinc on various forms of cholesterol as the actual amount of high zinc and the duration it is fed.

In summary, the present findings indicated that a moderately high intake of zinc (811  $\mu\text{mol/d}$ ; 53 mg/d) does not induce changes indicative of decreased-copper status or function in postmenopausal women fed low dietary copper (15.7  $\mu\text{mol/d}$ ; 1 mg/d).

However, inadequate zinc might be a nutritional stressor of copper metabolism and use in humans that might increase the need for copper above an intake of 15.7  $\mu\text{mol}$  (1 mg) of Cu/d for postmenopausal women. The findings also indicate that copper-status indicators might be useful for evaluating changes in the zinc status of humans.

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*(For an additional perspective, see Editorial Opinions.)*