Comparative Responses of Rats to Different Copper Intakes and Modes of Supplementation (43594)

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Abstract. **Purified diets deficient in copper and based on either sucrose, egg white, and corn oil or sucrose, casein, corn starch, and safflower oil were fed to young rats. Graded amounts of copper were supplied in drinking solutions with the former diet and by addition to the latter diet; anatomical, chemical, and physiologic responses were compared. Three μg Cu/ml and 5 μg Cu/g were sufficient to maximize the direct assessments of copper nutriture (copper in blood plasma, heart, and liver). Nutritional adequacy by indirect criteria (heart iron, plasma ceruloplasmin, heart weight divided by body weight, plasma cholesterol, and body weight) generally was found with 3 μg/ml and 4 μg/g. Anemia was an insensitive characteristic of deficiency. Liver iron was minimized by 4 μg Cu/ml and 5 μg Cu/g. Most of the differences in response to copper added to water in comparison to copper added to diet probably were explained by the lower amount of copper in the casein diet. Responses to the two dietary regimens were similar when variables were plotted against liver copper. Correlation coefficients with liver copper ranged from 0.52 for liver zinc to 0.96 for heart iron. Liver copper probably is the best index of copper nutriture.** [P.S.E.B.M. 1993, Vol 203]

Responses to diets low in essential nutrients can be extremely variable. In early experiments on egg white injury (biotin deficiency), György (1) studied more than 1300 rats and found that the diet usually produced skin lesions in 4–7 weeks. However, 60 rats proved refractory to the diet for 16 to 26 weeks. Williams (2) noted that pigeons used in bioassay of extracts efficacious in the treatment of beriberi "vary greatly in the rapidity with which they develop forthright symptoms." Our experience with copper deficiency has been similar. For example, in four apparently similar experiments, the time at which half the rats died as a result of eating a diet deficient in copper ranged from 40 to 96 days (3).

Biochemical and physiologic responses can be quite variable even within a single experiment. Among rats fed a diet deficient in copper, some rats lived 40–415 days after the time (73 days) at which 75% were dead of deficiency (L. M. Klevay, unpublished). Among nonanemic rats fed a diet deficient in copper (4), hepatic iron, which increased 3-fold, was the most sensitive sign of deficiency and decreased body weight (by 25%), the least sensitive of the characteristics evaluated. It can be seen that some animals and some nutritional indices are more resistant to copper deficiency than others.

The present experiments were prompted by the observation that an amount of copper sufficient in one experiment (5) to maintain plasma copper was insufficient in another (6). Similarly, ceruloplasmin was found to be low in a group of rats thought to be fed adequate copper (J. T. Saari, unpublished). Two commonly used methods of providing trace element supplementation were studied. Variability in response to graded doses of copper may be useful in consideration of similar variability in response to other nutrients.

Materials and Methods

Animals and Diets: Experiment 1. Thirty male, weanling, Sprague-Dawley rats (Harlan Sprague-Daw-

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ley, Madison, WI)[2] were fed a purified diet for 4 weeks which without supplementation was deficient in both copper and zinc. The diet (fed *ad libitum*) with added biotin (7) was based on 62% sucrose, 20% egg white, 10% corn oil (by weight), and Jones Foster salt mix without copper or zinc and contained all other nutrients known to be essential for rats (6, 8). Drinking solutions (*ad libitum*) were supplemented with 10 μg Zn/ml (as acetate) and either 0, 2, 3, or 4 μg Cu/ml (as sulfate) (8). Housing was similar to that described (9). Seven animals were assigned to the groups lower in copper and eight were assigned to the higher copper groups. Rats were assigned to groups so that mean weights were matched as closely as possible. Dietary copper was 0.98 μg/g.

Animals and Diets: Experiment 2. Fifty-four male, weanling Sprague-Dawley rats (Harlan Sprague-Dawley) were divided into weight-matched groups of nine. Each group was fed (*ad libitum*) for 5 weeks a purified diet (10), based on 39% sucrose, 20% casein, 20% cornstarch, 5% safflower oil, and AIN-76 salt mix without added copper, to which varying amounts of copper (0–5 μg/g of diet) had been added. Housing was similar to that for Experiment 1. Dietary copper was 0.22 μg/g.

Blood and Organ Analyses. Animals were weighed weekly, and were sacrificed under intraperitoneal sodium pentobarbital. Blood was collected before death by cardiac puncture with ammonium heparin or sodium EDTA as anticoagulant. Heart and one lobe of liver were dissected free, blotted, weighed, and frozen for subsequent analyses.

Plasma cholesterol was determined by the method of Allain *et al.* (11) using reagents available commercially (Sigma Diagnostics, St. Louis, MO). Serum ceruloplasmin was measured by the method of Schosinsky *et al.* (12) in Experiment 1 and by the method of Sunderman and Nomoto (13) in Experiment 2. Ceruloplasmin units from Experiment 2 (mg/dl) were converted to those from Experiment 1 (units/liter) by multiplying by 3.5, a conversion factor suggested by the study of Schosinsky *et al.* (12), who define a unit as the amount of ceruloplasmin that oxidized 1 μmol of *o*-dianisidine dihydrochloride per minute.

Freeze-dried organs and diet samples were digested with nitric acid-hydrogen peroxide; copper, iron, and zinc were measured by atomic absorption spectrophotometry (6). Elements in plasma were determined directly by graphite furnace.

Statistical Analysis. Data were analyzed by one-way analysis of variance (14) with dietary copper concentration (in water or diet) as the independent variable. Comparisons between means were done by Tukey's studentized range test (14). To determine whether a relation could be defined between liver Cu and the other variables, polynomial regression lines were calculated (14). The optimum fit for each polynomial was found for each variable by increasing the order of the polynomial until adding additional terms caused no significant improvement in the prediction. Significance of the correlation coefficients (R) was tested by calculation of $F = (R^2/k)/[(1-R^2)/(n-k-1)]$ (14), where n = number of points and k = order of polynomial regression. Variability comparisons were made by the binomial test (15).

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Results

Experiment 1. In general, assays were done on all animals. The notable exception resulted from the death of an unsupplemented rat with a ventricular aneurysm.

All variables except for liver zinc were changed by increasing copper in the drinking solution (Table I). Liver copper was increased significantly in rats given a drinking solution containing 2 μg/ml, was further increased by 3 μg/ml, but was not increased more by 4 μg/ml. Two micrograms of copper (per ml) in the drinking solution were sufficient to produce a significant increase in heart iron, hematocrit, and body weight and a significant decrease in heart weight/body weight and cholesterol. Larger concentrations did not produce further significant change. A significant change was produced by 3 μg/ml, in comparison to 2 μg/ml, for plasma copper, heart copper, ceruloplasmin, and liver iron. Only liver iron was changed (significantly) more by 4 μg/ml.

Experiment 2. All variables, including liver zinc, were changed by increasing copper in the diet (Table II). Some of the dose responses were rather flat, however. Heart weight/body weight decreased and hematocrit and body weight increased significantly when 1 μg Cu/g was added to the diet and remained unchanged with higher amounts of copper. Plasma cholesterol decreased with 3 μg Cu/g. Ceruloplasmin increased with the addition of 4 and 5 μg Cu/g. Heart iron also increased 4 μg Cu/g. Liver copper was the most responsive of the variables to dietary copper, increasing significantly with the addition of 1, 2, and 5 μg Cu/g; liver iron also decreased at the latter dietary concentration.

Data for the several variables were plotted against liver copper for Experiment 1 (Fig. 1) and Experiment 2 (Fig. 2). Inspection of these plots led to our impression that variability of these measurements generally was greatest in the groups unsupplemented with copper. Comparison of the coefficients of variation of the unsupplemented group with those of the group receiving the lowest copper supplement revealed that eight of 10 of the former coefficients in Experiment 1, and eight of nine in Experiment 2, were greater than those of the

Table I. Characteristics of Copper Deficiency, Experiment 1

Variable	Drinking solution copper[a] (μg/ml)				Analysis of variance
	0	2	3	4	*P*
Liver[b] Cu (μg/g)	1.0 ± 0.2*	5.2 ± 0.6†	10.3 ± 0.3‡	10.0 ± 0.2‡	<0.0001
Plasma Cu (μg/ml)	0.02 ± 0.004*	0.06 ± 0.02*	0.82 ± 0.05†	0.86 ± 0.02†	<0.0001
Heart Cu (μg/g)	3.8 ± 0.2*	6.9 ± 0.5*	16.7 ± 1.1†	16.1 ± 0.8†	<0.0001
Heart Fe (μg/g)	205 ± 13*	299 ± 8†	300 ± 5†	302 ± 9†	<0.0001
Liver Fe (μg/g)	778 ± 46*	657 ± 25*	515 ± 27†	394 ± 27‡	<0.0001
Liver Zn (μg/g)	72 ± 5	80 ± 4	82 ± 2	80 ± 1	0.16
Ceruloplasmin (units/liter)	1.6 ± 1.2*	4.4 ± 2.4*	96 ± 6†	100 ± 3†	<0.0001
Heart wt/body wt (mg/g)[c]	7.78 ± 0.51*	4.82± 0.08†	4.15 ± 0.07†	4.06 ± 0.06†	<0.0001
Hematocrit (%)	21 ± 4*	39 ± 1†	38 ± 1†	37 ± 1†	<0.0001
Cholesterol (mg/dl)	144 ± 8*	115 ± 4†	110 ± 4†	105 ± 3†	<0.0001
Body wt (g)	177 ± 11*	211 ± 3†	218 ± 4†	214 ± 4†	<0.0001

[a] Data are expressed as mean ± SE. Values in a row with different symbols (*, †, ‡) are different ($P < 0.05$).
[b] All organ analyses are dry weight.
[c] Heart weight ÷ body weight.

Table II. Characteristics of Copper Deficiency, Experiment 2

Variable	Added dietary copper[a] (μg/g)						Analysis of variance
	0	1	2	3	4	5	*P*
Liver[b] Cu (μg/g)	0.9 ± 0.1*	4.9 ± 0.4†	7.8 ± 0.5‡	9.0 ± 0.3‡	9.5 ± 0.7‡	11.2 ± 0.2§	<0.0001
Heart Cu (μg/g)	5.2 ± 0.3*	7.5 ± 0.3*	11.0 ± 0.7†	12.6 ± 0.5†	14.0 ± 1.0†	17.6 ± 1.0‡	<0.0001
Heart Fe (μg/g)	222 ± 11*	271 ± 14*,†	251 ± 18*,†	291 ± 24*,†	298 ± 15†	283 ± 13*,†	<0.04
Liver Fe (μg/g)	561 ± 52*	542 ± 44*	452 ± 24*	507 ± 40*	465 ± 29*	298 ± 28†	<0.0001
Liver Zn (μg/g)	74 ± 4*	83 ± 2*,†	76 ± 2*	84 ± 2†	80 ± 2*,†	79 ± 1*,†	<0.02
Ceruloplasmin (units/liter)	11 ± 1*	19 ± 1*	25 ± 5*,†	21 ± 2*	45 ± 10†	87 ± 7‡	<0.0001
Heart wt/body wt[c] (mg/g)	5.6 ± 0.4*	4.0 ± 0.1†	3.8 ± 0.1†	3.6 ± 0.1†	3.5 ± 0.1†	3.5 ± 0.1†	<0.0001
Hematocrit (%)	23 ± 2*	43 ± 1†	45 ± 1†	45 ± 1†	45 ± 1†	44 ± 1†	<0.0001
Cholesterol (mg/dl)	115 ± 10*	104 ± 5*,†	97 ± 5*,†	82 ± 6†	85 ± 5†	80 ± 6†	<0.001
Body wt (g)	243 ± 6*	289 ± 5†	288 ± 8†	295 ± 8†	301 ± 6†	291 ± 7†	<0.0001

[a] Data are expressed as mean ± SE. Values in a row with different symbols (*, †, ‡, §) are different ($P < 0.05$).
[b] All organ analyses are dry weight.
[c] Heart weight ÷ body weight.

latter group. The respective probabilities by the binomial test are <0.05 for Experiment 1, <0.02 for Experiment 2, and (16 of 19) <0.002 for both experiments combined.

Because the plots had similar appearances even though the copper supplements were given by drinking solution (Experiment 1) and by diet (Experiment 2), data for both experiments were merged and polynomial regressions on liver copper were calculated (Table III). All regressions were highly significant ($P < 0.001$), with the coefficients of determination (R^2) ranging from 0.26 for liver zinc and heart iron to 0.93 for plasma copper.

Discussion

Copper deficiency in rats unsupplemented with copper was verified in these experiments by several criteria. Anemia has been associated with diets deficient in copper since copper first was shown to be an essential nutrient by Hart *et al.* (16). Although anemia is not an inevitable consequence of copper deficiency (4, 17, 18), its presence in association with a diet low in copper is sufficient for diagnostic purposes. Early death (3, 19) in association with ventricular aneurysm (20) is common in copper deficiency. The cardiac enlargement found here has been found many times by others (21, 22). Since the first association between the metabolism of copper and that of cholesterol (8), hypercholesterolemia from copper deficiency has been found in at least 13 independent laboratories (23, 24). Although a couple of apparently well-controlled experiments in copper deficiency have failed to produce hypercholesterolemia (24), hypercholesterolemia in copper deficiency generally is accepted according to Prohaska (25).

These and most of the other measurements reported here are indirect indications of copper deficiency. They are nonspecific as other nutritional and toxic insults can affect them. However, when two groups of animals differing only in copper intake are being compared, inference of deficiency based on the presence of one or more of these characteristics is likely to be correct.

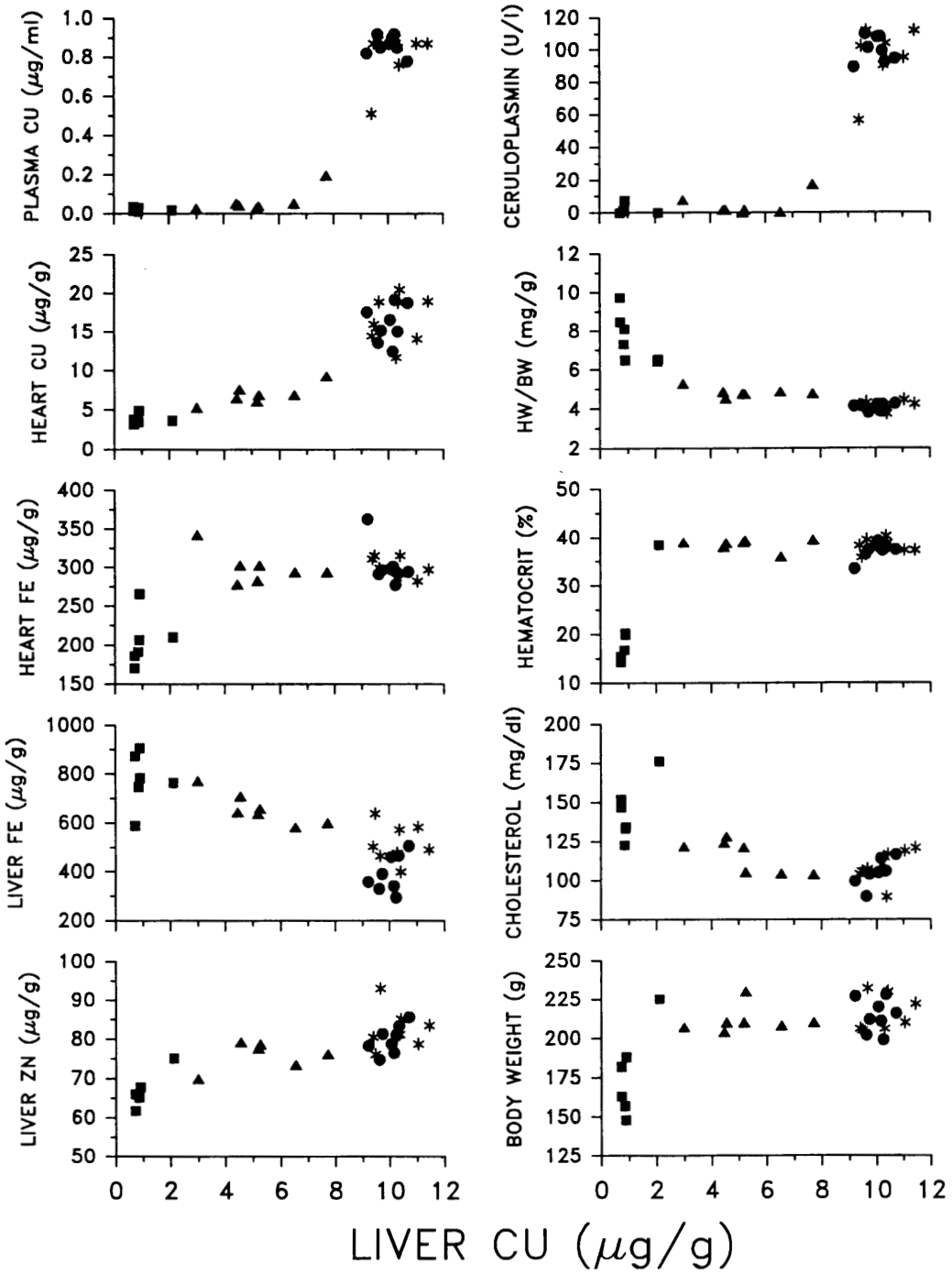

Figure 1. Values of 10 variables used as signs of Cu deficiency plotted against liver Cu (μg/g dry wt) for different amounts of copper in the drinking solution (Expt. 1). The amount of copper added is indicated by these symbols: ■, 0; ▲, 2; *, 3; and ●, 4 μg/ml of drinking water. These same symbols were used for corresponding amounts in Figure 2.

Three direct assessments of copper status are reported here: copper in blood plasma, heart, and liver. Copper was low in all of these in unsupplemented rats. There was no significant increment in these measurements after the copper concentration in the drinking solution reached 3 μg/ml (Experiment 1). Plasma copper was not measured in Experiment 2; diet at 5 μg Cu/g produced significant increments in liver and heart copper in comparison to 4 μg/g.

All of the indirect assessments except liver iron revealed adequate status when copper reached 3 μg/ml in Experiment 1. Similar adequacy was found in Experiment 2 at 4 μg/g; again liver iron was an exception. This slight difference between the two experiments may be explained by the lower dietary copper in Experiment 2. That higher copper intakes are needed to minimize liver iron has been reported (4).

Because the liver is "the central organ in copper

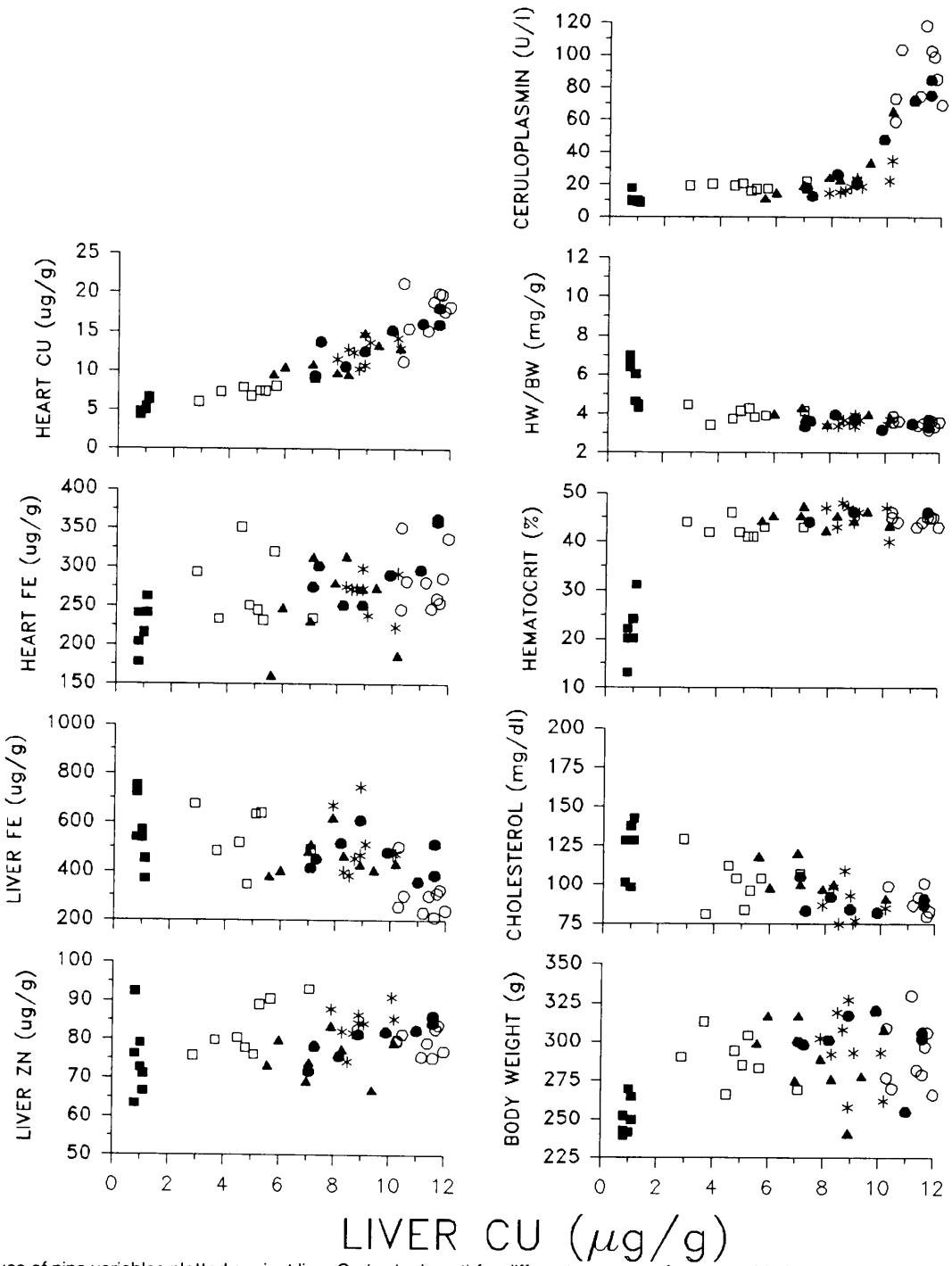

Figure 2. Values of nine variables plotted against liver Cu (μg/g dry wt) for different amounts of copper added to the diet (Expt. 2). The amount of copper added to the diet is indicated by these symbols: ■, 0; □, 1; ▲, 2; *, 3; ●, 4; ○, 5 μg/g of diet.

metabolism" (26), we have calculated regressions of all other variables on liver copper. It appears from the figures that liver copper in the 9–11 μg/g range is associated with adequate copper nutriture according to the other criteria. It seems prudent to recall the warnings of Mertz, Underwood, and Vallee about placing too much emphasis on mineral concentrations in assessing nutritional adequacy, however (27–30). As noted above, anemia is not a particularly sensitive index of copper deficiency as anemia was eliminated at the lower concentrations of liver copper associated with the lower copper intakes.

The inverse correlation between liver copper and cholesterol in plasma has been found many times (31–34). As the liver is the main organ of cholesterol synthesis (35) and excretion (36), and hepatic hydroxymethylglutaryl-coenzyme A reductase activity is increased in copper deficiency (37, 38), liver copper is

Table III. Regressions on Liver Copper (μg/g dry wt)[a]

Variable	Polynomial regression coefficients[b]				Coefficient of determination[c]
	a_0	a_1	a_2	a_3	(R^2)
Plasma Cu	0.201	−0.231	0.051	−0.002	0.93
Heart Cu	4.27	0.206	0.087		0.86
Hematocrit	9.50	14.6	−1.98	0.083	0.81
Ceruloplasmin	15.5	−8.94	1.40		0.71
Heart wt/body wt[d]	7.96	−1.55	0.194	−0.008	0.70
Body wt-1	147	33.0	−4.99	0.237	0.66
Liver Fe	702	−27.0			0.41
Body wt-2	228	28.3	−3.73	0.152	0.35
Cholesterol	129	−3.76			0.34
Heart Fe	205	17.6	−0.894		0.26
Liver Zn	69.4	2.18	−0.103		0.26

[a] Data from both experiments were merged, except for body weight because rats were larger in Experiment 2. Plasma copper was measured only in Experiment 1.
[b] Least-squares, best-fit coefficients from the equation: variable = $a_0 + a_1(Cu_L) + a_2(Cu_L)^2 + a_3(Cu_L)^3$, where Cu_L = liver Cu concentration (μg/g dry wt).
[c] All of the coefficients of determination (correlation coefficient squared) listed are significant ($P < 0.001$, F test).
[d] Heart weight ÷ body weight.

more likely to control the concentration of plasma cholesterol than it is to control the values of the other variables more closely associated mathematically (Table III). Some of these significant regressions on liver copper are more likely to be indirect.

These associations (Table III) explain 26–93% of the variance of these variables, with the direct assessments of copper status being at the top of the list. The existence of these associations probably is more important than whether they are linear, quadratic, or cubic. To our knowledge, these associations have not been noticed by others. The general smoothness of the data fit indicates that responses to copper are similar even when both diets and method of supplementation are different. Prohaska and Lukasewycz (39) observed similar correlation ($r = 0.66$, $P < 0.01$) between liver copper and the plaque-forming response of cultured splenocytes of mice to sheep erythrocytes. Similarly, Morin *et al.* (40) found correlations ($r = -0.5$, $P < 0.025$) between liver copper and thromboxane synthesis by blood platelets. It is incorrect to infer from the large coefficient that plasma copper is as good as liver copper in the assessment of nutriture because several other experiments have shown normal (41) or high plasma copper when organ copper is low (42–46). Low plasma copper does indicate deficiency, however.

Just as individual animals (and presumably, people) respond variably to a particular nutritional insult such as copper deficiency, various characteristics used in the assessment of nutriture exhibit different sensitivities of response to dietary copper. Some are optimized at lower intakes than others. Variability of response seemed greater at low copper intakes. Liver copper probably is the best of the direct indices of nutriture; so far, organ analysis in assessment of human nutriture has found only limited application (47, 48). Some other direct indices and some indirect indices are closely associated with liver copper. Some of these associations may be causal; most probably are indirect. Variability in responses to dietary copper probably are not unique. It seems likely that responses to other nutrients are variable as well.

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