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Responses of rat cuproenzymes to variable dietary copper

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Weanling, 19-day-old, male Sprague-Dawley rats were offered a basal diet low in copper (Cu), 0.40 mg/kg (6.3 nmol/g), and drinking water containing 0, 0.5, 1, or 10 (Cu-adequate) mg Cu/L. After 30 days, plasma and heart cuproenzymes were assayed. Body weight and hemoglobin were equivalent among groups. Rats drinking 1 mg Cu/L did not have elevated heart/body weight, lower liver Cu, or higher liver Fe compared with Cu-adequate rats. However, activity of two blood enzymes (plasma ceruloplasmin and serum peptidylglycine α-amidating monooxygenase (PAM)) and three heart enzymes (cytochrome c oxidase, Cu, Zn-superoxide dismutase, and PAM) were lower compared with rats supplemented with 10 mg Cu/L. Groups drinking 0.5 mg Cu/L or deionized water had even lower activities including that of heart dopamine-β-monooxygenase. Serum and heart PAM activities patterned one another, suggesting that serum PAM may be a suitable marker of tissue Cu status. Addition of $CuSO_4$ to the PAM assay increased the apparent activity in a manner inversely related to dietary Cu intake in both heart and serum samples. The Cu stimulation index (activity with added Cu/activity with basal Cu) may also be a useful tool to assess Cu status. (J. Nutr. Biochem. 8:316–321, 1997) © *Elsevier Science Inc. 1997*

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Introduction

The essential nature of dietary copper (Cu) has been recognized since the 1920s.[1] Despite continuous research on Cu there is currently no specific recommended dietary allowance (RDA) for humans in the US. There is an estimated safe and adequate daily dietary intake (ESADDI) recommended by the National Research Council ranging between 1.5 and 3.0 mg/day.[2] There are many factors that are responsible for the lack of an RDA, and these were summarized recently.[3] One anomaly pointed out in that discussion was the disparity between the current ESADDI and the data provided by Klevay that approximately one-third of 849 diets analyzed contain <1 mg Cu.[3] There is no evidence of overt Cu deficiency in the US. Further work in establishing the Cu requirement for humans clearly is needed. One of the limitations in this endeavor has been the lack of suitable biochemical indicators that track Cu status. Several factors from blood (plasma ceruloplasmin, erythrocyte superoxide dismutase, platelet cytochrome *c* oxidase, plasma copper, hemoglobin, and immune status) have been examined with limited success.[4,5] It would be useful to identify additional sensitive biochemical markers of human Cu status.

In contrast with human adults, dietary Cu has a pronounced and predictable outcome when studied in rodents.[6] Postmortem analyses have verified and extended the essential nature of Cu for all biological systems. Most notably, recent studies indicate that the consequences of perinatal Cu deficiency may not be reversible. Even after 5 months of Cu repletion, offspring of Cu-deficient dams exhibited abnormal behavior.[7] The only measured biochemical variable different between the control rats and Cu-repleted rats was lower brain Cu concentrations in the latter group. Marginal Cu deficiency in rats (intake approximately one-half of that recommended) resulted in perceptible abnormalities of the immune system and cardiovascular system without change in the usual biochemical indicators of Cu status.[8,9] There is a clear need to identify good indicators of marginal Cu status.

The purpose of the current studies was to determine the biochemical response of variable Cu intake in growing rats. A commonly used strain was used (Sprague-Dawley), as was a diet used by many nutritional scientists (AIN-76).

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Several cuproenzymes were measured (ceruloplasmin (CPL), cytochrome *c* oxidase (CCO), dopamine-β-monooxygenase (DBM), peptidylglycine-α-amidating monooxygenase (PAM), and Cu,Zn-superoxide dismutase (SOD)) with the goal of identifying a sensitive predictor of Cu status. Growth, hemoglobin concentration, and liver mineral analyses were also determined to assess Cu status. PAM activity was measured in both serum and heart to determine if there was any correlation between the two sampling sites. Responses of PAM activity to a series of variable Cu intakes were not reported previously. Because PAM can be sassayed using small samples of serum, it might serve as a useful clinical marker of Cu status. The current rat model was designed to investigate that possibility.

Methods and materials

Animals and diets

Rat dams and offspring were offered a Cu-deficient purified diet (Teklad Laboratories, Madison, WI USA) and either low Cu drinking water or Cu-supplemented drinking water. The purified diet was formulated according to the AIN-76A diet and contained the following major components (g/kg diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; AIN-76A vitamin mix, 10; DL-methionine, 3; choline bitartrate, 2; and ethoxyquin, 0.01.[10,11] Cupric carbonate was omitted from the AIN-76 mineral mix, resulting in a modified AIN-76A diet containing 0.40 mg Cu/kg (6.3 nmol/g). Diet and drinking water were available ad libitum. Rats were maintained at 24°C with 55% relative humidity on a 12-hr light cycle (0700–1900) in an AAALAC-accredited facility. All protocols were formally approved by the University of Minnesota Animal Care Committee.

Pregnant Sprague-Dawley rats were purchased commercially (Harlan Sprague-Dawley, Indianapolis, IN USA) and 2 days after parturition litter size was adjusted to eight pups. Eight dams were divided into two equal groups and were given either 2 mg Cu/L (Cu-2) or 20 mg Cu/L (Cu-20) by adding Cu to the drinking water as $CuSO_4$. One male and one female rat from each of the eight dams was killed at 19 days of age to assess Cu status. Remaining pups were weaned at 19 days and some were transferred to stainless steel cages and housed individually for 30 days. Sixteen male offspring from four litters of Cu-adequate dams, drinking 20 mg Cu/L, were randomly divided into four groups. All rats were offered the Cu-deficient diet and drank either deionized water (Cu-0), or that water supplemented with 0.5 mg Cu/L (Cu-0.5), 1.0 mg Cu/L (Cu-1), or 10 mg Cu/L (Cu-10). The later group were considered Cu-adequate controls. Female offspring from the group Cu-20 dams were used for another purpose. Remaining pups from the group Cu-2 dams were subjected to euthanasia.

Sample collection

Rats were sampled at ages 19 days and 49 days. Blood samples were drawn into heparinized microhematocrit tubes from trunk blood after decapitation after a light ether anesthesia. A small aliquot was also removed for hemoglobin analysis. Additional blood was collected in plastic tubes and allowed to clot. Plasma and serum were obtained by centrifugation. Livers were removed, rinsed with deionized water, weighed, and a portion processed for metal analysis. Hearts were removed, rinsed in deionized water, and blotted before weighing. For enzymatic analysis, hearts were homogenized for 30 seconds in 9 volumes of 0.05 mol/L potassium phosphate (pH 7.0) with a Tissumizer and microprobe (SDT-080EN, Tekmar Co., Cincinnati, OH USA).

Chemical analyses

Portions of liver and 1-g samples of diets were wet-digested with 4 mL of concentrated HNO_3 (AR select grade, Mallinckrodt, St. Louis, MO USA), and the residue was brought to 4.0 mL with 0.1 mol/L HNO_3. Samples were then analyzed for Cu and Fe by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT USA).

Hemoglobin was determined spectrophotometrically as metcyanhemoglobin.[12] Total protein content was determined by analysis of the heart homogenates using a modified Lowry method with bovine albumin as a reference.[13]

Enzymatic analyses

More extensive details of the enzyme assays are described elsewhere. Plasma was obtained from microhematocrit tubes after centrifugation and the activity of the cuproprotein CPL (EC 1.16.3.1) was determined by measuring the ability of plasma to oxidize *o*-dianisidine using a modification, 37°C, of the method of Lehman et al.[14] CCO activity was determined on fresh homogenates as loss of activity upon storage was noted previously.[15] Homogenates were treated with 0.1% Triton X-100. Initial velocity was measured at 25°C and the rate of ferricytochrome *c* formation (μmol/min) was determined using a molar extinction coefficient of 19,600 for reduced-oxidized cytochrome *c*. DBM activity was determined spectrophotometrically on heart homogenates, as described previously for mouse brain by measuring conversion of tyramine to octopamine.[16] PAM activity was measured as previously described, where the trinitrophenyl-labelled substrate and product were separated by reverse phase HPLC, with the effluent monitored at 350 nm (Kratos 757 UV detector, Applied Biosystems, Foster City, CA USA).[17] Serum PAM assays were conducted for 2 hr at 37°C using 10 μL serum and no detergent. PAM activity in heart homogenates and serum was also determined in the presence of exogenous Cu^{2+}, 5 μmol/L $CuSO_4$, and a Cu stimulation index (Cu SI) was calculated (Cu SI = activity with added Cu/activity with basal Cu). Basal Cu content of the PAM reaction mixture was 0.25 μmol/L. Reactants were fractionated on a 4.6 × 150 mm analytical column (Ultrasphere-ODS, 5 μm, Beckman Instruments, Fullerton, CA USA) preceded by a 3.2 × 15 mm guard column (Aquapore ODS 7 μm, Applied Biosystems). Peak heights were recorded (Omniscribe recorder, Houston Instruments) and picomoles of product were calculated by comparison with trinitrophenyl-D-Tyr-Val-NH_2. SOD activity was measured by following inhibition of pyrogallol autooxidation at 320 nm as described previously.[12] Homogenates were treated with 0.4 volumes of chloroform:ethanol (15:25) to inactivate manganese SOD. Kinetic enzyme assays were run in duplicate with a temperature controlled spectrophotometer (Beckman DU-8). Activities, with the exception of plasma ceruloplasmin and serum PAM, were all expressed per mg heart protein.

Statistical analyses

The effect of maternal Cu intake on offspring characteristics were analyzed by Student's *t* test, $\alpha = 0.05$, using a Macintosh computer and statistical software (Statview 4.5, Abacus Concepts, Inc., Berkeley, CA USA). Statistical evaluation of different Cu doses was done using one-way ANOVA and Fisher's PLSD test, $\alpha = 0.05$. Variance equality was evaluated by the *F*-test. Enzyme data were normalized (% mean value from Cu-10 group) so that comparisons between different enzymes as well as doses could be compared.

Table 1 Features of 49-day-old male sprague-dawley rats

Characteristic	Supplemental Copper (mg/L) 0	0.5	1	10	ANOVA (*P*)
Body weight (g)	219 ± 8.4	230 ± 3.8	236 ± 10.1	226 ± 5.8	NS
Heart/body (mg/g)	5.29 ± 0.11[a]	5.18 ± 0.12[a]	4.75 ± 0.08[b]	4.50 ± 0.14[b]	.0013
Hemoglobin (g/L)	122 ± 5.0	132 ± 6.2	126 ± 5.8	121 ± 1.4	NS
Liver Cu (nmol/g)	17.7 ± 1.3[c]	33.6 ± 3.3[b]	52.3 ± 2.0[a]	51.8 ± 6.4[a]	.0001
Liver Fe (μmol/g)	2.40 ± 0.16[a]	1.51 ± 0.45[b]	1.38 ± 0.09[b]	0.96 ± 0.09[b]	.0096

Values are means ± SEM (n = 4). For those characteristics with an ANOVA $P < 0.05$ means were compared by Fisher's PLSD test. Means not sharing a common superscript letter were different, $P < 0.05$. Details of the dietary treatments are listed in the Methods. The basal dietary Cu level was 6.3 nmol/g. This diet and various supplemental drinking water was offered beginning at 19 days of age.

Results

Most characteristics of the offspring derived from dams on the Cu-2 and Cu-20 treatments were similar at age 19 days (data not shown). The mean body weight ± SEM for the eight males sampled was 45.8 ± 2.4. The mean plasma activity of the males from the Cu-2 dams was 65.2 ± 0.86 units/L (n = 4) and not different than the CPL activity of males from the Cu-20 dams 62.9 ± 2.84 (n = 4). There was marked difference, however, in liver Cu stores. The males from Cu-2 dams averaged 260 ± 23.3 nmol/liver and the males from Cu-20 dams 576 ± 58.2 nmol/liver, more that 2.2 fold higher $P < 0.01$. A similar pattern was observed for female offspring (data not shown). Because of this, it was decided that the postweanling study should be conducted with only Cu-adequate offspring and so males from the Cu-20 dams were used to continue the dose-response experiment.

The initial body weight of the 16 male rats ranged between 40.9 and 49.3 g and averaged 45.4 ± 0.6. After 30 days of treatment eating Cu-deficient diet supplemented with water containing 0, 0.5, 1.0, or 10.0 mg/L Cu, rats were killed and several characteristics were determined (*Table 1*). Rats grew about 180 g during this period and no differences in body weight between groups were detected. Liver weight was not affected by varying Cu intake (data not shown). Heart weight and relative heart weight was higher in the rats drinking water with the two lowest Cu levels (*Table 1*). Although the degree of cardiac hypertrophy was rather modest compared to some studies, it was still evident in the Cu-0.5 supplemented group. We failed to detect any differences in hemoglobin concentrations due to varying Cu intake (*Table 1*). Hematocrits were also not different between groups ranging in value between 44.1 and 45.5%. Liver Cu concentration reflected Cu intake (*Table 1*). The concentration in the Cu-0 group was the lowest and about half that measured in the Cu-0.5 group. Supplementing the water with 1.0 mg Cu/L elevated liver Cu about 1.5 fold to a level measured in the control group (Cu-10). Liver Fe concentration was higher in the Cu-0 group than in any of the supplemented groups (*Table 1*). There was high variability in liver Fe levels in the groups receiving the lowest Cu intake.

CPL activity was determined on thawed plasma samples taken from microhematocrit tubes and PAM activity was determined on fresh serum taken from the blood of decapitated rats (*Figure 1*). CPL activity was barely detectable in plasma from the Cu-0 and Cu-0.5 groups. Activity in plasma from the Cu-1 group was about 30% lower than activity in rats from the Cu-10 group; notably, liver Cu was equivalent in these two supplemented groups (*Table 1*). CPL activity in the Cu-10 group averaged 116 units/L. PAM activity of serum in the Cu-10 group averaged 335 pmol/(h · mL). The pattern of PAM activity in the other three groups was similar to the CPL pattern except that activity in the Cu-0 and Cu-0.5 groups were 20 and 35%, respectively, of the activity in the Cu-10 group (*Figure 1*). The activity in the Cu-1 group was about two-thirds that of the Cu-10 group.

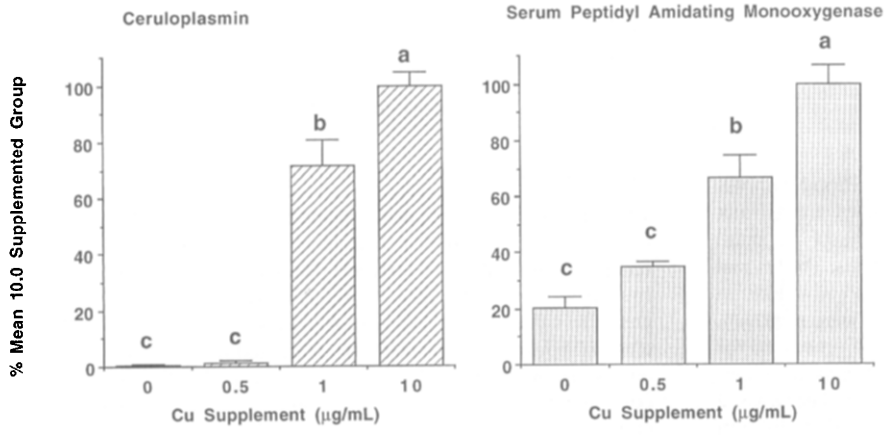

Figure 1 Plasma ceruloplasmin and serum peptidyl-α-amidating monooxygenase activity of 49d old male Sprague Dawley rats. Weanling rats were offered a Cu-deficient diet and variable Cu in their drinking water for 30 days. Individual data were normalized (%) to the mean value of the group drinking 10 μg Cu/mL. Bars represent means ± SEM (n = 4). Means not sharing a common superscript letter were different $P < 0.05$.

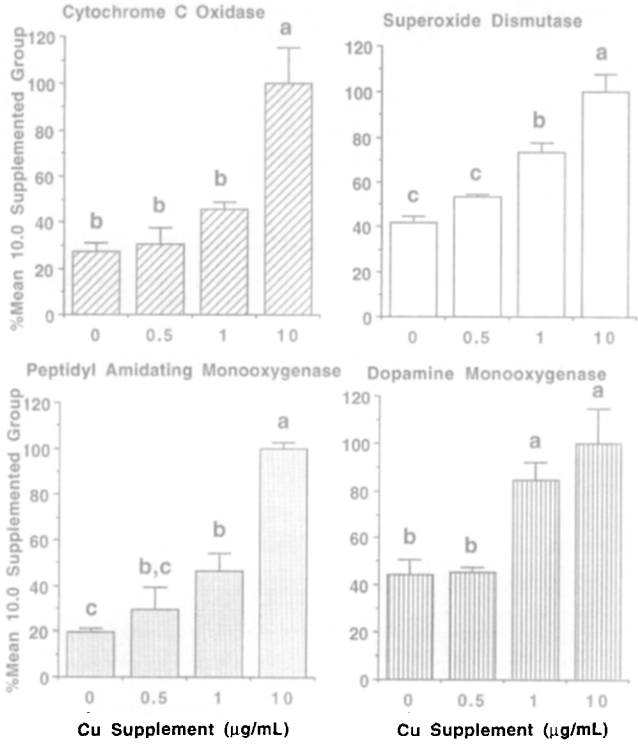

Figure 2 Heart cytochrome c oxidase, superoxide dismutase, peptidyl-α-amidating monooxygenase, and dopamine-β-monooxygenase activity of 49-day-old male Sprague-Dawley rats. Weanling rats were offered a Cu-deficient diet and variable Cu in their drinking water for 30 days. Individual data were normalized (%) to the mean value of the group drinking 10 μg Cu/mL. Bars represent means ± SEM (n = 4). Means not sharing a common superscript letter were different $P < 0.05$.

Cuproenzyme activity of hearts was influenced by dietary Cu intake as well (*Figure 2*). Activity of heart CCO in the Cu-10 group averaged 2.49 μmol/(min · mg protein). Activity in the hearts of the other three groups, drinking water with less Cu, was markedly lower. Even in the Cu-1 group, the average heart CCO activity was less than half that of the Cu-10 rats (*Figure 2*). Heart SOD of the Cu-10 group averaged 79.2 units/mg protein. Activity in the Cu-10 control group exceeded that in the three other groups. Heart SOD activity of the Cu-1 group was only 75% that of the Cu-10 group (*Figure 2*). Heart PAM activity in the Cu-10 group averaged 353 pmol/(h · mg) and exceeded the activity in the other three groups in a manner similar to that of CCO and SOD (*Figure 2*). Activity in the Cu-1 group was less than half that of the Cu-10 group. Heart DBM activity was also determined and averaged 0.33 nmol/(h · mg) in the Cu-10 group. Activity in the Cu-0 and Cu-0.5 groups was less than half that of the Cu-10 group. No significant difference between DBM activity in the Cu-10 and Cu-1 groups was detected (*Figure 2*). Heart protein concentrations ranged between 114 and 135 mg/g and were not different between groups.

Previous estimates of DBM activity in brain and adrenal gland of Cu-deficient rats indicated higher rather than lower activity in homogenates in vitro.[18] Therefore, DBM activity in heart was reassayed in the presence of exogenous 5 μM $CuSO_4$. Activity in the Cu-0 group increased 6.6-fold compared to the DBM activity without added Cu. This stimulation was higher than the 2.6-fold elevation measured in the Cu-10 group, $P < 0.05$. There was no difference in the mean heart DBM activity between the two groups when assayed with added Cu^{2+}.

Cu stimulation index (Cu SI) values were also determined for both serum and heart PAM for all four groups (*Table 2*). After the addition of 5 μM Cu^{2+}, PAM activity in the serum was equivalent in all four groups ranging between 1.16 and 1.24 nmol/(h · mL). This was because the Cu SI values were inversely proportional to the dietary Cu intake (*Table 2*). The Cu SI values of serum PAM clearly discriminated between Cu-deficient (Cu-0 and Cu-0.5 groups) compared to Cu-adequate (Cu-10). There was no discriminatory power between the Cu-1 and Cu-10 groups. A similar pattern for heart PAM Cu SI values was observed (*Table 2*). After Cu^{2+} addition, the heart PAM activity was equivalent in all four groups ranging between 1.6 and 2.1 nmol/(h · mg). The heart PAM Cu SI values reflected dietary Cu intake in a manner similar to serum PAM (*Table 2* and *Figure 1*).

Discussion

Clinical consequences of dietary Cu deficiency in human adults are uncommon.[19] Detection of marginal Cu deficiency is currently difficult if not impossible. Danks suggested to assay the oxidase activity of plasma CPL before and 3 days after a physiological Cu supplement.[19] This approach would be useful in assessing the lack of adequate

Table 2 Activity and copper stimulation index (Cu SI) of peptidylglycine-α-amidating monooxygenase (PAM)

Measurement	Supplemental Copper (mg/L)				
	0	0.5	1.0	10.0	ANOVA (P)
Serum-PAM (nmol/h · mL)	1.18 ± 0.08	1.24 ± 0.10	1.19 ± 0.07	1.16 ± 0.08	NS
Serum-PAM (Cu SI)	19.4 ± 3.75[a]	10.8 ± 1.36[b]	5.65 ± 0.91[c]	3.45 ± 0.21[c]	.0006
Heart-PAM (nmol/h · mg)	1.86 ± 0.13	2.06 ± 0.07	1.88 ± 0.15	1.60 ± 0.10	NS
Heart-PAM (Cu SI)	27.3 ± 2.77[a]	25.7 ± 6.14[a]	12.5 ± 2.39[b]	4.58 ± 0.36[b]	.0019

PAM activity was determined in the presence of exogenous 5 μmol/L Cu^{2+} and the ratio of that activity to basal activity (0.25 μmol/L Cu^{2+}) was determined and is referred to as the copper stimulation index (Cu SI). Values are means ± SEM (n = 4) from tissues of 49d old male Sprague Dawley rats. Means were compared by Fisher's PLSD test for those measurements with a significant ($P < 0.05$) ANOVA. Means not sharing a common superscript letter were different, $P < 0.05$. Details of the dietary treatments are listed in the methods and materials section. The basal dietary Cu level was 6.3 nmol/g. This diet and various supplemental drinking water was offered beginning at 19 days of age.

liver Cu to support CPL holoenzyme secretion. Liver Cu concentration is the best predictor of Cu status, but liver biopsy generally would be too invasive unless there were compelling clinical reasons. Milne has argued, based on several human studies, that serum Cu and CPL may not be satisfactory for assessing marginal Cu deficiency.[20] He suggests that more than one measurement is needed. The rodent model system used in the current experiments assumed that rats and humans respond biochemically in a similar manner and shows that many of the heart and blood cuproenzymes might be appropriate to detect variable Cu intake. In particular, the data show that serum PAM, basal activity, and the Cu SI correlate with Cu intake. This assay is an attractive noninvasive complement to CPL activity for the assessment of Cu status in rodents, and perhaps in humans. Our CPL assay requires 15 μL, and our basal PAM and stimulated PAM each 10 μL of sample, a total amount that could be obtained from a single microhematocrit tube. The source of serum PAM is not known with certainty, but Mains et al. offer some evidence for a neuronal basis.[21]

Other cuproenzyme candidates for assessment of Cu status include CCO and SOD.[19,20] Cells from the white cell population such as platelets and neutrophils have been used to measure CCO with some success. This assay is not trivial and requires fresh cells and is highly dependent on the cytochrome *c* concentration. This somewhat diminishes comparisons among various labs. SOD is a more stable enzyme. However, as shown in the current experiments and others, SOD is generally less sensitive to changes in Cu status than CCO.[15] Some have used erythrocyte SOD activity to assess Cu status, however, the slow turnover of the red cell population would seem to make this choice not as suitable for acute changes. DBM activity changed in the hearts of the Cu-deficient rats in the current studies in support of earlier work on catecholamine content. Serum DBM may be useful to assess humans after dietary Cu deficiency. It will be important in future studies to include both DBM activity and catecholamine concentrations since there are certain cases were both are needed to interpret the results.[16,18] The current studies in Sprague-Dawley rats exposed two situations in which liver Cu and CPL activity did not agree, underscoring Milne's idea that multiple indicators of Cu status are needed. The first instance was in 19-day offspring of dams drinking supplemented water with two compared to 20 mg Cu/L. Recall that CPL activity was equivalent in the two groups, whereas liver Cu was 2.2 fold higher in offspring nursed by dams on the 20 mg/L supplement. The second situation was in the dose-response study in which rats in the Cu-1 and Cu-10 groups had equivalent liver Cu concentrations but the CPL activity of the Cu-1 group was 30% lower than the Cu-10 group. CPL is an acute phase protein and subject to induction by multiple stimuli. It seems that several indicators of Cu status are needed scientifically to establish a correlation with Cu intake. Blood rather than urine seems appropriate for this assessment.[19]

Can cuproenzyme activities be used to help establish the Cu requirement for humans? That approach is certainly employed in recent human studies.[4,20] Based on balance studies, the adult human Cu requirement was estimated to be 1.5 mg.[22] More recent studies indicate no detectable changes in Cu status or in salivary or urinary Cu in young men consuming half that amount of Cu.[23] Perhaps as more sensitive indicators of Cu status are developed the goal of assessing marginal Cu intake can be reached. Studies in rodents suggest that the dietary level of Cu needed to ensure adequate growth and development is about 5 to 6 mgk/g. Presumably, less is required by nongrowing adults. How does this compare with diets humans consume? Assuming that the lower limit of the ESADDI is sufficient for adults to ensure adequate Cu status, the 1.5 mg of Cu within a 2500 kcal diet composed of 17% protein, 38% fat, and 45% carbohydrate with a moisture content of 65% would correspond to a Cu concentration of about 1 mg/kg. I hypothesize that feeding adult rats a diet containing 1 mg/kg Cu would result in animals with Cu status difficult to diagnose without postmortem analyses. In the current studies, rats in the Cu-0.5 and Cu-1 groups were still growing and this perhaps explains why some of the cuproenzyme assessments were successful in distinguishing between those groups and the Cu-adequate controls (Cu-10).

Results of the current experiments should be interpreted with caution, because they were done with a single diet and single gender and a single strain. Many pioneer researchers showed that variables such as protein type can affect Cu status.[24] Using the same diet as in the current studies using Holtzman rather than Sprague-Dawley male rats results in mild growth impairment and anemia.[17] Status indicators also can change in marginal Cu deficiency as the duration of the study is increased.[25] It is interesting to note, however, how similar the Cu requirement of rats is compared to other mammals such as mice, cats, and pigs.[26–28] Significance of the rat model in developing further assessment tools, especially cuproenzymes from the blood, will require further research and evaluation.

The robust stimulation of PAM activity by adding Cu^{2+} to extracts from Cu-deficient rats is somewhat unusual for cuproenzymes. CPL, SOD, and CCO activity of Cu-deficient rats did not respond significantly to in vitro addition of cupric sulfate (unpublished data). It is known from tracer studies that DBM exchanges Cu rapidly.[29] The catalytic domain of DBM and PAM are highly homologous.[30] PAM, therefore, may also exchange Cu rapidly. This could explain the significant Cu SI for both DBM and PAM observed in our samples from Cu-deficient rats.

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