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Journal of Nutritional Biochemistry 16 (2005) 682–692

Journal of Nutritional Biochemistry

Serum superoxide dismutase 3 (extracellular superoxide dismutase) activity is a sensitive indicator of Cu status in rats☆

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Received 16 December 2004; received in revised form 25 February 2005; accepted 14 March 2005

Abstract

Sensitivity of the assay for Cu,Zn superoxide dismutase 3 (SOD3), the predominant form of SOD in serum, can be increased, and interferences caused by low-molecular-weight substances in the serum can be reduced by conducting the assay at pH 10 with xanthine/xanthine oxidase and acetylated cytochrome *c* (cyt *c*) as superoxide generator and detector, respectively. Serum SOD3 activity was assayed under these conditions in an experiment where weanling, male rats were fed diets for 6 weeks containing 3, 5 and 15 mg Zn/kg with dietary Cu set at 0.3, 1.5 and 5 mg Cu/kg at each level of dietary Zn. Serum SOD3 responded to changes in dietary Cu but not to changes in dietary Zn. A second experiment compared serum SOD3 activity to traditional indices of Cu status in weanling, male and female rats after they were fed diets containing, nominally, 0, 1, 1.5, 2, 2.5, 3 and 6 mg Cu/kg for 6 weeks. Serum SOD3 activity was significantly lower ($P<.05$) in male rats fed diets containing 0 and 1 mg Cu/kg and female rats fed diet containing 0 mg Cu/kg compared with rats fed diet containing 6 mg Cu/kg. These changes were similar to changes in liver Cu concentrations, liver cyt *c* oxidase (CCO) activity and plasma ceruloplasmin in males and females. Serum SOD3 activity was also strongly, positively correlated with liver Cu concentrations over the entire range of dietary Cu concentrations ($R^2=.942$ in males, $R^2=.884$ in females, $P<.0001$). Plots of serum SOD3 activity, liver Cu concentration, liver CCO activity and ceruloplasmin as functions of kidney Cu concentration all had two linear segments that intersected at similar kidney Cu concentrations (18–22 μg/g dry kidney in males, 15–17 μg/g dry kidney in females). These findings indicate that serum SOD3 activity is a sensitive index of Cu status.

Keywords: Superoxide dismutase 3; Serum; Copper status; Copper indices; Rats

1. Introduction

Superoxide dismutases (SODs) belong to a family of antioxidant enzymes that catalyze the dismutation of superoxide to yield hydrogen peroxide and oxygen. Two SODs are expressed intracellularly in eukaryotic cells. Cu,Zn SOD (SOD1), the major intracellular form of SOD, is located in the cytoplasmin and nucleus. The other intracellular SOD, MnSOD (SOD2), is compartmentalized primarily in the mitochondrial matrix. A third SOD, termed extracellular SOD (SOD3), is expressed in the extracellular matrix and on cell surfaces and is the predominant SOD in extracellular fluids. Although SOD1 and SOD3 are distinctly different in terms of molecular structure and cellular location, they both require Cu and Zn for enzymatic activity [1].

Copper deficiency decreases the activity of SOD1 in a variety of tissues in animals [2–5] and its activity in erythrocytes is a putative biomarker for Cu status in humans [6,7]. However, SOD3 activity has not received much attention as a potential index of Cu status. A few studies have reported reduced activities of SOD3 in the plasma or serum of rats consuming severely copper-deficient diets [8–10] and the study by DiSilvestro [8] showed that serum SOD3 may be reduced in rats consuming marginally Cu-deficient diets. This finding suggests that SOD3 activity in serum may be a useful biochemical indicator of Cu status.

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doi:10.1016/j.jnutbio.2005.03.009

The lack of a standard assay limits the use of serum SOD3 activity as a biomarker of Cu status. SOD activity is frequently assayed with indirect assays that involve the additions of a superoxide generator and a detector whose reaction with superoxide is spectrophotometrically monitored. The presence of SOD in a sample inhibits the reaction between superoxide and the detector by competing for the superoxide supplied by the generator. The degree of inhibition is proportional to the activity of SOD in the sample. Several superoxide generators and detectors have been used in combination to assess in vitro SOD activity. The results from these assays are often variable and conflicting when the activity of SOD3 is measured in bodily fluids. A comparison of several indirect assays of SOD3 in body fluids concluded that the assay measuring inhibition of pyrogallol autooxidation, in which pyrogallol serves as both the source and sensor of superoxide, is subject to fewer interferences from substances in the sample than assays using xanthine/xanthine oxidase (X/XO) as the source of superoxide [11]. However, the assay employing X/XO as the superoxide generator and oxidized cytochrome *c* (cyt *c*) as the detector has advantages for measuring SOD3 activity in fluids such as serum or plasma where SOD3 activity is low. A key advantage is the greatly increased sensitivity of the assay when it is conducted with sodium carbonate buffer at pH 10 and acetylated cyt *c* as the superoxide detector. In addition, fewer interferences resulting from reoxidation of reduced cyt *c* occur when acetylated cyt *c* is used as the superoxide detector [12].

The current estimated average requirement (EAR) and recommended dietary allowance (RDA) for Cu are 0.7 and 0.9 mg/day, respectively, for adults [13]. Examination of the usual intakes of Cu compiled in the National Health and Nutrition Examination Survey III, Continuing Survey of Food Intakes by Individuals II and Total Diet Studies provides no consistent estimate of how well population estimates of Cu intakes correspond to the EAR and RDA for Cu. However, these epidemiological data indicate that up to 25% of men and 50% of women consume diets that fall below the EAR for Cu and up to 50% of men and 75% of women consume diets that fall below the RDA for Cu, depending on age, pregnancy and lactation (see Tables C-15, D-2 and E-3 in Ref. [13]). Other data indicate that Cu intakes fall below the EAR and RDA for 11% and 25%, respectively, of the population consuming a Western diet [14] and for 36% and 62%, respectively, for randomly selected subjects in Maryland [15]. Collectively, these data suggest that marginal Cu deficiency in humans is not a rare occurrence and may be an underrecognized nutritional problem. An important aspect of studying marginal Cu deficiency is finding a practical, sensitive index of Cu status. Accordingly, the objective of the present study was to determine if serum SOD3 activity, measured at pH 10 with X/XO and acetylated cyt *c*, can be used to assess marginal Cu status in rats.

2. Methods and materials

2.1. Animals and diets

2.1.1. Experiment 1

An initial experiment was performed to examine the effects of several combinations of dietary Cu and Zn concentrations on serum SOD3 activity. Basal diet for this experiment was based on the AIN-93 G composition [16] formulated with sprayed egg white as the protein source. Cu and Zn levels were adjusted by adding with $CuSO_4 \cdot 5H_2O$ and $ZnCO_3$ to the basal diet to obtain diets having 0.3 mg of supplemental Cu/kg with 3, 5 or 15 mg of supplemental Zn/kg; 1.5 mg of supplemental Cu/kg with 3, 5 or 15 mg of supplemental Zn/kg; 3 mg of supplemental Cu/kg with 3, 5 or 15 mg of supplemental Zn/kg; and 5 mg of supplemental Cu/kg with 3, 5 or 15 mg of supplemental Zn/kg. Analysis of the 12 diets by flame atomic absorption spectroscopy indicated that the Cu and Zn concentrations were 0.63±0.08 mg Cu/kg, 3.37 mg Zn/kg; 0.64±0.09 mg Cu/kg, 5.7±0.64 mg Zn/kg; 0.84±0.03 mg Cu/kg, 16.99±0.36 mg Zn/kg; 1.79±0.14 mg Cu/kg, 3.27 mg Zn/kg; 1.88±0.01 mg Cu/kg, 5.99±0.55 mg Zn/kg; 1.80±0.18 mg Cu/kg, 17.9±0.96 mg Zn/kg; 3.45±0.11 mg Cu/kg, 3.62±0.28 mg Zn/kg; 3.31±0.23 mg Cu/kg, 5.32±0.15 mg Zn/kg; 3.20±0.06 mg Cu/kg, 17.19±0.37 mg Zn /kg; 4.85±0.18 mg Cu/kg, 3.11±0.26 mg Zn/kg, 5.35±0.16 mg Cu/kg, 5.78±0.02 mg Zn/kg; 4.99±0.10 mg Cu/kg, 17.08±0.19 mg Zn/kg. Weanling, male Sprague–Dawley rats (Charles River, Wilmington, MA) were divided into 16 weight-matched treatment groups each having 7 rats. Each group was fed ad libitum 1 of the 12 diets described above for 6 weeks. For each dietary Cu level, a group of rats was pair-fed with diet containing 15 mg Zn/kg at the amount consumed by the group fed diet containing 3 mg Zn/kg.

2.1.2. Experiment 2

A second experiment was performed to examine the effects of marginal Cu intake on serum SOD3 activity. Basal diet for this experiment was also based on the AIN-93 G composition [16] formulated with casein as the protein source. Dietary Cu levels were adjusted by either adding no supplemental Cu to the basal diet or by adding $CuSO_4 \cdot 5H_2O$ in graded amounts to obtain diets having, nominally, 0, 1, 1.5, 2, 2.5, 3 or 6 mg Cu/kg. Analysis of the diets by flame atomic absorption spectroscopy indicated that the diets contained (mean±S.D.) 0.30±0.01, 0.90±0.07, 1.35±0.11, 1.84±0.19, 2.23±0.11, 2.65±0.13 and 5.28±0.24 mg Cu/kg. Assays for mineral content of the diets in this experiment and the first experiment were validated by simultaneous analysis of a durum wheat flour reference standard (No. 8436, National Institute of Standards and Technology, Gaithersburg, MD) and a dietary reference standard (HNRC-2A) developed by the Grand Forks Human Nutrition Research Center. Analyzed values for Cu and Zn in the standards fell within the certified range for Cu and Zn, thus validating the

assayed values for Cu and Zn in the diets. Weanling male and female Sprague–Dawley rats were divided into seven weight-matched groups that were fed one of the seven diets ad libitum for 6 weeks.

In both experiments, the rats were housed in stainless steel cages in a room maintained at 22±2°C and 50±10% humidity with a 12-h light/dark cycle. After the rats consumed their respective diets for 6 weeks, they were anesthetized with ketamine/xylazine and blood was withdrawn from the inferior vena cava and organs were removed for analysis. The study was approved by the Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center, and the rats were maintained in accordance with the National Research Council Guidelines for the care and use of laboratory rats.

2.2. Analytical methods

The left lobe of the liver and kidneys from each rat were rinsed with cold (4°C) 0.15 mol/L NaCl and lyophilized. The lyophilized liver and kidney samples were ashed by using a previously reported method [17] and their Cu concentrations were measured by inductively coupled plasma-emission spectroscopy (Optima 3100 XL, Perkin Elmer, Waltham, MA). The Cu analysis was validated by simultaneous assay of a bovine liver reference sample (No. 1577a, National Institute of Standards and Technology). Analyzed values for Cu in the standard fell within the certified range for Cu, thus validating the assayed values for liver and kidney Cu concentrations.

Liver remaining after the removal of the left lobe was used for the assay of cyt *c* oxidase (CCO) activity. The liver samples were homogenized in 10 volumes of buffer containing 0.25 M sucrose, 0.1 mM EGTA and 10 mM HEPES, pH 7.4. CCO activity was measured in the liver homogenates by monitoring the loss of ferrocytochrome *c* at 550 nm (Model DU-600 spectrophotometer, Beckman Instruments, Palo Alto, CA), as previously described [4].

After blood was withdrawn from the inferior vena cava, a portion was transferred to a tube containing 4.5% EDTA (30 μl/ml blood) for hematological analysis. Hemoglobin concentrations, hematocrits and red cell distribution widths were measured with an electronic cell counter (Cell-Dyne 3500, Abbott Diagnostics, Santa Clara, CA). Ceruloplasmin and SOD3 activities were assayed in serum collected from the portion of blood not treated with EDTA. Ceruloplasmin activity was assayed by its *p*-phenylenediamine oxidase activity [18] and SOD3 activity was assayed by monitoring the inhibition of acetylated cyt *c* reduction at pH 10.0, as previously described [12,19].

To determine their relative proportions in rat sera, SOD1 and SOD3 were separated by passing serum through a concanavalin A–Sepharose column, as previously described [19]. SOD activity was measured in the bound and unbound fractions after the fractions were concentrated with a centrifugal filter device (Centiprep YM-30, Millipore Corp., Bedford, MA). The bound and unbound fractions also were analyzed by Western blotting for the presence of SOD1 and SOD3. Samples, which were reduced with dithiothreitol, were subjected to SDS-PAGE (15 μg protein/lane, 12% polyacrylamide gels) and electroblotted to polyvinylidene fluoride membrane (Immobilon-P, Millipore Corp.). The immunoblots were incubated for 1 h with either mouse anti-SOD3 antibody (a gift from Dr. Oury, University of Pittsburgh) or rat anti-SOD1 antibody (Stressgen Biotechnologies, Victoria, British Columbia, Canada). Both antibodies were diluted 1:1000. After incubation with primary antibody, the blots were incubated with horseradish peroxidase-coupled rabbit anti-IgG. The immunoreactive bands were visualized by chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ) with an imaging densitometer (EpiChemi^3, UVP, Upland, CA).

Protein concentrations in liver and serum samples were determined with bicinchoninic acid (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL).

2.3. Statistics

Unless otherwise indicated, data are reported as means±S.D. Data were analyzed by two-way ANOVA to determine the effects of dietary Cu and Zn and their interaction in the first experiment and the effects of dietary Cu and sex and their interaction in the second experiment. Individual means were tested by pairwise comparison with Tukey's multiple comparison test when main effects and

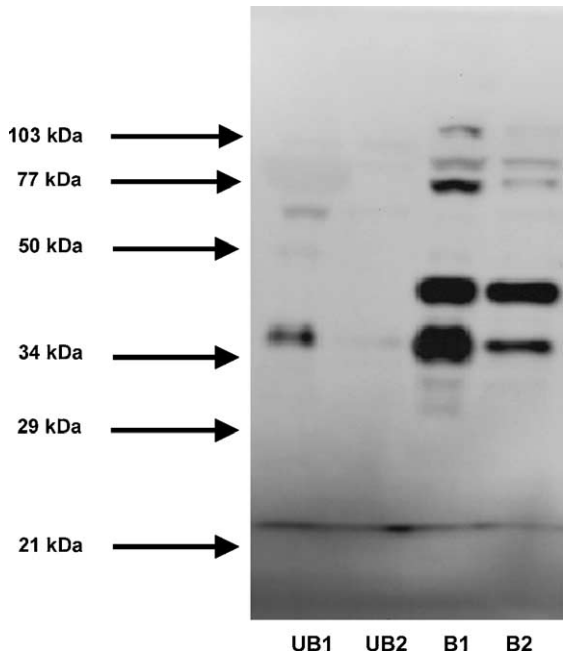

Fig. 1. Western blot analysis of rat serum SOD3. Lanes 1 and 2 represent proteins collected in two fractions (UB1 and UB2) that did not bind to a conconavalin A–Sepharose chromatography column. Lanes 3 and 4 represent proteins that bound to the column that were eluted in two fractions (B1 and B2) by α-methylmannoside. Each lane of the SDS-PAGE gel from which the blot was produced contained 15 μg of protein.

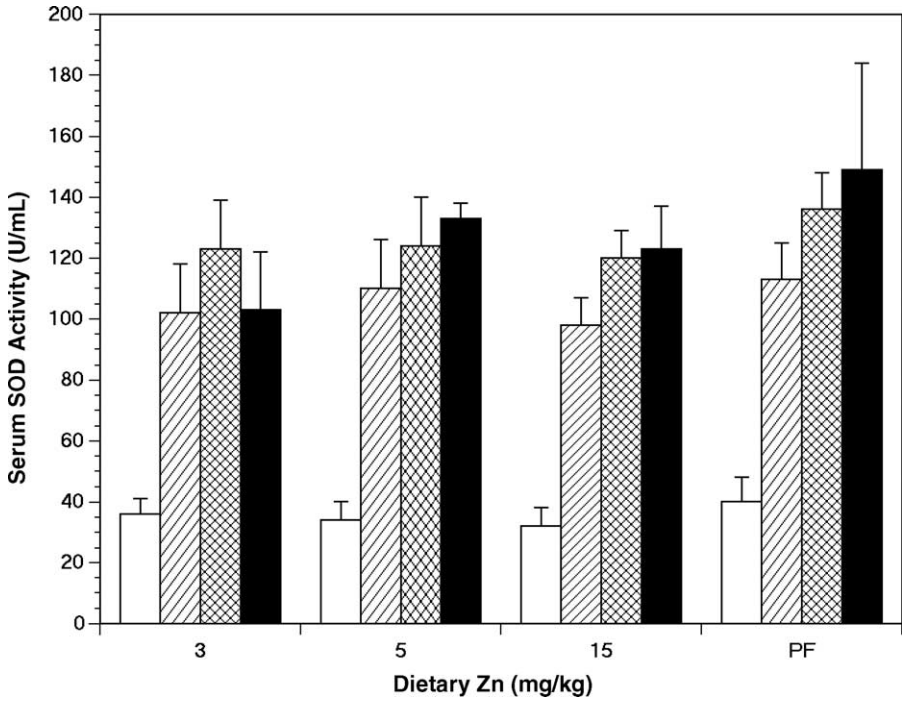

Fig. 2. The effects of dietary Cu and Zn contents on serum SOD3 activity. Male rats were fed diets containing 3, 5 and 15 mg Zn/kg that also contained 0.3 mg Cu/kg (open bars), 1.5 mg Cu/kg (diagonal hatched bars), 3 mg Cu/kg (cross hatched bars) or 5 mg Cu/kg (black bars). For each dietary Cu concentration, food intakes of pair-fed rats (PF) fed diets containing 15 mg Zn/kg were adjusted to the food intakes of rats fed diets containing the corresponding Cu concentrations and 3 mg Zn/kg. *P* values for the effects of dietary Zn, dietary Cu and Dietary Cu×Zn interaction from 2-way ANOVA were .001, <.0001 and .17, respectively. A unit of SOD activity is the amount of enzyme that causes 50% inhibition in the reduction of acetylated ferricytochrome *c* by superoxide generated by X/XO at pH 10 and 25°C.

interactions were significant. Differences were considered significant at $P<.05$. Linear relationships between indicators of Cu status were analyzed by linear regression analysis [20]. A nonlinear, two-phase regression model was utilized to estimate change points following the parameterization described by Bacon and Watts [21]. The correlation between kidney Cu and dietary Cu concentrations was determined by using the analyzed values for dietary Cu concentrations.

3. Results

Preliminary measurements of serum SOD3 activity were made by using serum from adult, male rats fed a

Table 1
The effect of dietary Cu on liver and kidney Cu concentrations, liver CCO activity and plasma ceruloplasmin

Dietary Cu (mg/kg)	Liver Cu (μg/g dry liver)		Kidney Cu (μg/g dry kidney)		Liver CCO (U/mg protein)*		Ceruloplasmin (mg/dL)	
	Male	Female	Male	Female	Male	Female	Male	Female
0	2.0±0.9[a]	2.9±1.1[a]	10.3±0.4[a]	10.0±0.8[a]	0.033±0.017[a]	0.094±0.044[a,**]	6.3±0.05[a]	ND***
1	7.8±2.3[b]	13.8±1.3[b,*]	14.5±1.5[a,b]	17.7±2.7[a,b]	0.056±0.022[a]	0.232±0.046[b,**]	14.3±8.5[a,b]	37.1±8.0*
1.5	10.8±2.4[c]	13.8±1.8[b,*]	17.6±3.2[b,c]	22.8±7.7[b,c]	0.075±0.013[a,b]	0.227±0.024[b,**]	28.3±14.0[b,c]	40.6±10.5
2	12.9±1.7[c,d]	14.6±1.7[b]	21.9±5.8[b,c,d]	23.2±5.3[b,c,d]	0.111±0.025[b]	0.241±0.021[b,**]	35.6±6.3[c,d]	31.9±19.1
2.5	14.1±0.5[d]	14.4±0.9[b]	27.3±4.0[c,d]	28.0±8.8[c,d]	0.113±0.024[b]	0.230±0.031[b,**]	39.5±7.6[c,d]	42.4±4.1
3	13.6±0.5[d]	14.8±1.4[b]	29.4±10.1[d,e]	31.7±9.6[d,e]	0.106±0.029[b]	0.237±0.024[b,**]	41.4±5.7[c,d]	44.9±4.6
6	14.2±0.9[d]	16.0±1.1[b]	37.4±15.1[e]	34.7±11.1[e]	0.110±0.028[b]	0.231±0.018[b,**]	44.0±5.5[d]	44.0±11.2
ANOVA	*P*		*P*		*P*		*P*	
Cu	<.0001		<.0001		<.0001		<.0001	
Sex	<.0001		.34		<.0001		.01	
Cu×Sex	<.0001		.86		<.0001		.0002	

Values are means±S.D. Means in a column that do not share a superscript are significantly different ($P<0.05$, Tukey's multiple comparison test). For kidney Cu, the superscripts indicate significant differences between the pooled means for males and females because the Cu×Sex interaction was not significant.

* A unit of CCO activity is the amount of enzyme that oxidizes 1 μmol ferrocytochrome *c*/min at 30°C.

** Difference between males and females at a given dietary Cu is significant ($P<.05$, Tukey's multiple comparison test).

*** Not detectable.

Table 2
The effects of dietary Cu on hemoglobin, hematocrit, red cell distribution width and heart weight

Dietary Cu mg/kg	Hemoglobin concentration (g/L)		Hematocrit		Red cell distribution (width %)		Heart weight (g)	
	Male	Female	Male	Female	Male	Female	Male	Female
0	114±17	131±2	0.37±0.04	0.40±0.01	18.3±3.5	15.3±1.6	1.52±0.30[a]	0.74±0.05
1	126±6	136±11	0.40±0.02	0.41±0.02	13.7±1.0	13.9±0.7	1.07±0.13[b]	0.69±0.08
1.5	123±8	137±10	0.39±0.02	0.42±0.02	14.2±0.9	13.3±0.4	1.10±0.09[b]	0.70±0.04
2	121±9	140±8	0.39±0.02	0.42±0.03	16.3±3.8	13.2±1.2	1.08±0.09[b]	0.71±0.09
2.5	128±7	144±8	0.41±0.02	0.43±0.03	14.9±1.7	13.5±0.8	1.07±0.12[b]	0.68±0.07
3	127±5	140±14	0.40±0.02	0.41±0.02	14.0±0.6	14.0±0.1	1.06±0.15[b]	0.71±0.05
6	127±6	138±9	0.40±0.02	0.42±0.03	14.4±1.1	14.0±0.7	1.04±0.11[b]	0.68±0.06
ANOVA	*P*		*P*		*P*		*P*	
Cu	.02		.03		.0006		<.0001	
Sex	.0001		<.0001		.002		<.0001	
Cu×Sex	.86		.51		.10		<.0001	

Values are means±S.D. Means in a column that do not share a superscript are significantly different ($P<.05$, Tukey's multiple comparison test).

nutritionally adequate diet (AIN-93 with 6 mg Cu/kg and 15 mg Zn/kg) to determine if conducting the assay at pH 10 with X/XO and acetylated cyt *c* reduced interferences caused by low-molecular-weight substances in serum and increased sensitivity. SOD3 activity was measured in six rat serum samples before and after the enzyme was inactivated in a boiling water bath for 1.5 min. SOD3 activities (mean±S.D.) before and after heat inactivation were 104±12 and 12±4 U/ml serum, respectively. In another set of six samples from adult, male rats, SOD3 activity was 120±8 U/mL when assayed with the X/XO, acetylated cyt *c* system at pH 10 and 25±1 U/mL when assayed by monitoring the inhibition of pyrogallol, as previously described [4]. These results indicate that interference caused by the reduction of acetylated ferricytochrome *c* by low-molecular-weight substances in serum was minimal and sensitivity was increased when serum SOD3 activity was assayed using xanthine/xanthine oxidase as the superoxide generator and acetylated cyt *c* as the detector at pH 10.0. Also, no SOD3 activity was detected when xanthine oxidase was omitted from the reaction mixture, indicating that acetylated ferricytochrome *c* was not reduced by rat serum unless superoxide was generated in the reaction mixture. In addition, SOD3 activity in serum also was completely inhibited by 1 mM KCN, indicating that a Cu,Zn SOD was completely responsible for the activity.

Elution of rat serum from the concanavalin A–Sepharose chromatography column was accomplished in a stepwise manner [19]. Four fractions were collected by eluting the column with 3 mL of equilibration buffer (unbound 1), followed by 10 mL of equilibration buffer (unbound 2), followed by 5 mL of 0.5 M α-methylmannoside (bound 1) and finally with an additional 5 mL of 0.5 M α-methylmannoside (bound 2). Of the total activity applied to the column (24.5 U), 95% (23.4 U) was recovered, with 43% (10.1 U) in the unbound fractions and 57% (13.3 U) in the bound fractions. As shown in Fig. 1, SOD3 was detectable as a 40-kDa protein in the unbound 1 fraction and as 40- and 46-kDa proteins in the bound fractions. These results are consistent with the subunit molecular weight of rat SOD3, which exists as a dimer with an approximate molecular weight of 85 kDa [22]. Furthermore, the doublet bands representing immunoreactive proteins of close molecular weights in the bound fractions on our Western blots are similar to the pattern of doublet bands detected on Western blots of mouse lung SOD3 [23]. No immunoreactive SOD1 was detectable in either the unbound or bound fractions (data not shown). These data indicate that SOD3 is the major contributor to serum SOD activity in rats.

Fig. 2 shows the effects of dietary Cu and Zn on serum SOD3 activity (Experiment 1). Two-way ANOVA performed on these data indicated that dietary Cu and dietary Zn both significantly affected serum SOD3 activity. SOD3 activity was not significantly affected by interaction between dietary Cu and Zn, indicating that dietary Cu and Zn exerted their effects independently of one another. The mean serum SOD3 activities (mean±S.E.M.) for rats fed diets containing 3, 5 and 15 mg of supplemental Zn/kg and the pair fed group were 91±4, 100±3, 93±4 and

Table 3
The effect of dietary Cu on serum SOD3

Dietary Cu (mg/kg)	Serum SOD3 (U/mL)*	
	Male	Female
0	20±7[a]	40±13[a]
1	60±21[b]	137±20[b,**]
1.5	94±20[c]	137±14[b,**]
2	95±18[c]	148±26[b,**]
2.5	112±4[c]	161±8[b,**]
3	105±8[c]	146±12[b,**]
6	109±6[c]	147±11[b,**]
ANOVA	*P*	
Cu	<.0001	
Sex	<.0001	
Cu×Sex	.0001	

Values are means±S.D. Means in a column that do not share a superscript are significantly different ($P<.05$, Tukey's multiple comparison test).

* A unit of SOD activity is the amount of enzyme that inhibits the reduction of acetylated ferricytochrome c by 50%.

** Difference between males and females at a given dietary Cu is significant ($P<.05$, Tukey's multiple comparison test).

109±3 U/mL, respectively. However, the only statistically significant differences ($P<.05$) in activity occurred because the activity was higher in the pair-fed group compared with the groups fed diet containing 3 and 15 mg of supplemental Zn/kg. Serum SOD3 activity was not significantly different in rats fed diets containing 3, 5 or 15 mg of supplemental Zn/kg. The mean serum SOD3 activities (mean±S.E.M.) for rats fed diets containing 0.3, 1.5, 3 and 5 mg of supplemental Cu/kg were 35±3, 105±3, 126±4 and 127±4 U/mL, respectively. The activities in rats consuming diet containing 0.3 and 1.5 mg of supplemental Cu/kg were significantly different from one another and also were significantly lower than activities in rats consuming diet containing 3 and 5 mg of supplemental Cu/kg ($P<.05$).

Table 1 shows the variations in several biochemical indices of Cu status (liver Cu, kidney Cu, liver CCO activity and serum ceruloplasmin) with dietary Cu (Experiment 2). Two-way ANOVA performed on these data indicated that liver Cu, liver CCO activity and serum ceruloplasmin activity were influenced by a significant interaction between dietary Cu and sex. Liver Cu, CCO activity and serum ceruloplasmin activity were significantly lower only in

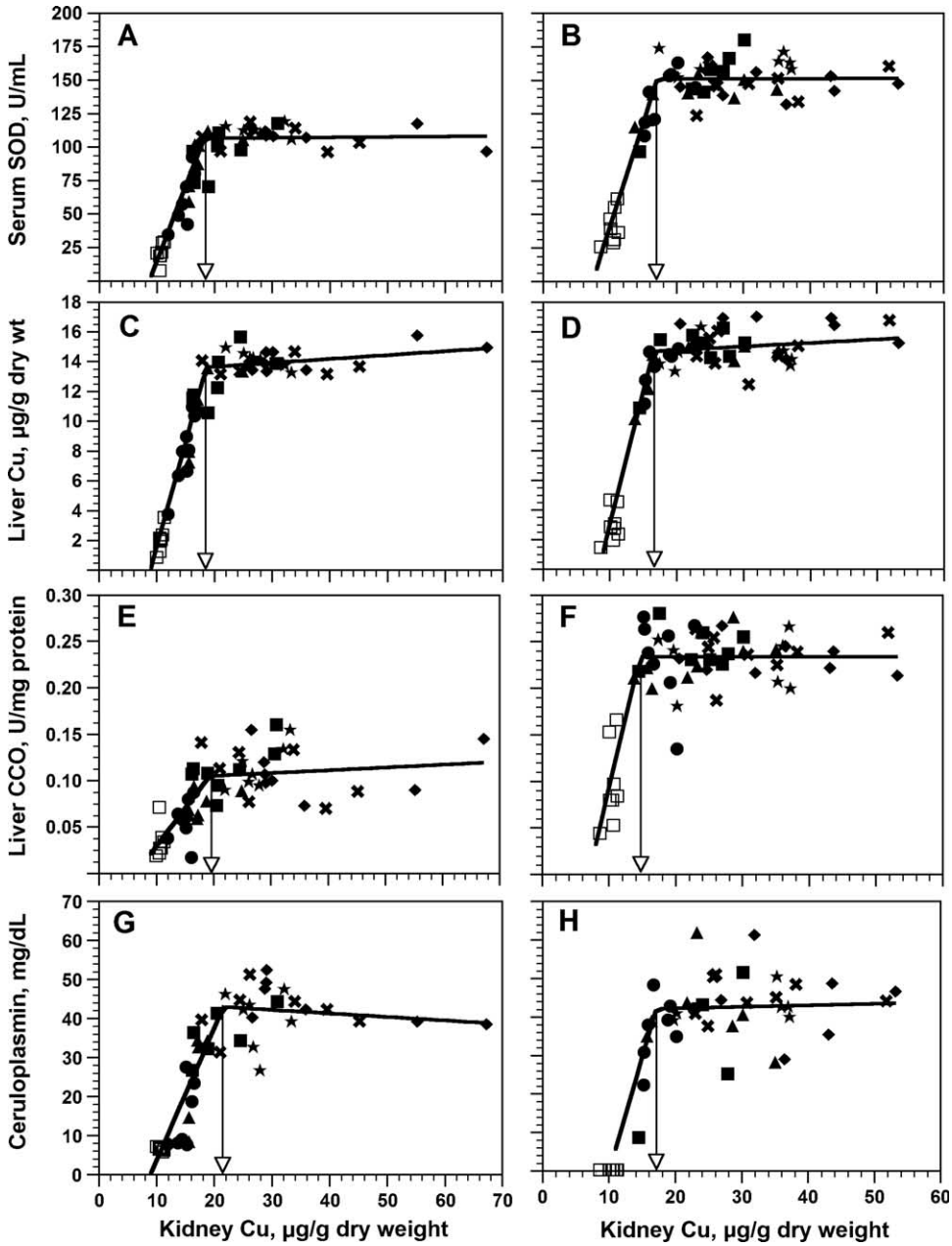

Fig. 3. Serum SOD3 activity, liver Cu concentration, liver CCO activity and ceruloplasmin as functions of kidney Cu concentration in male (panels A, C, E and G) and female (panels B, D, F and H) rats fed graded amounts of dietary Cu. The Cu concentrations in the various diets are represented as follows: 0.3 (open square), 1 (circle), 1.5 (triangle), 2 (black square), 2.5 (star), 3 (cross), and 6 mg Cu/kg (diamond). The arrow indicates the kidney Cu concentration at the point where the two segments of each plot intersect. A unit of SOD activity is defined in the legend to Fig. 2.

females consuming diet containing 0 mg Cu/kg compared with females consuming diet containing 6 mg Cu/kg. In males, liver Cu and ceruloplasmin activity were significantly lower in the rats consuming diets containing 0, 1 and 1.5 mg Cu/kg compared with males consuming diet containing 6 mg Cu/kg. Liver CCO activity in males consuming diets containing 0 and 1 mg Cu/kg was significantly lower than in males consuming diet containing 6 mg Cu/kg. However, liver CCO activity was higher in females than in males at each dietary Cu level. Kidney Cu was significantly affected by dietary Cu but not by sex nor by an interaction between dietary Cu and sex. Averaged over both sexes, kidney concentrations (mean±S.E.M.) in rats fed diets containing 0, 1, 1.5, 2, 2.5, 3 and 6 mg Cu/kg were 10.2±1.9, 16.1±1.9, 20.2±1.9, 22.6±1.9, 27.6±1.9, 30±1.9 and 36.0±1.9 μg Cu/g dry kidney, respectively. Kidney Cu concentrations in rats consuming diets containing 2.5 mg Cu/kg and below were significantly lower ($P<.05$) than those in rats consuming diet containing 6 mg Cu/kg.

Table 2 shows the effects of dietary Cu on hematological indices and heart weight. Two-way ANOVA performed on these data indicated that dietary Cu had a significant effect on hemoglobin concentrations and hematocrits regardless of sex. However, the only significant differences in hemoglobin concentrations and hematocrits occurred between rats consuming diets containing 0 and 2.5 mg Cu/kg. Hemoglobin concentrations and hematocrits in rats (male and female) consuming diet having 0 mg Cu/kg were 122±3 g/L and 0.38±0.01, respectively, compared with 136±3 g/L and 0.42±0.01, respectively, in rats consuming diet containing 2.5 mg Cu/kg ($P<.05$). Red cell distribution width in rats (male and female) consuming diet containing 0 mg Cu/kg was 16.8±0.5%, which was significantly higher ($P<.05$) than the red cell distribution widths in all other diet treatment groups. Heart weights were significantly affected by an interaction between dietary Cu and sex. Heart weights were significantly higher in males consuming diet containing 0 mg Cu/kg than in males from any other diet treatment group. In contrast, dietary Cu had no significant effect on heart weights in females.

Table 3 shows the effect of dietary Cu on serum SOD3 activity. Two-way ANOVA performed on these data indicated that serum SOD3 activity was significantly affected by an interaction between dietary Cu and sex. Serum SOD3 activity was significantly lower ($P<.05$, Tukey's multiple comparison test) in males consuming diets containing 0 and 1 mg Cu/kg compared with males consuming diet containing 6 mg Cu/kg. However, serum SOD3 activity in females was significantly lower only in the group consuming diet having 0 mg Cu/kg. Serum SOD3 activity also was significantly higher in females than in males except in the rats consuming diet containing 0 mg Cu/kg where differences in serum SOD3 activity between males and females were not significant. Regardless of the differences in serum SOD3 activity between males and females, the activity and liver Cu concentrations were positively correlated in males and females over the entire range of dietary Cu levels. The regression equations were SOD3=5.70 +7.32×liver Cu ($R^2=.94$, $P<.0001$) in males and SOD3=0.88+8.84×liver Cu ($R^2=.88$, $P<.0001$) in females.

Fig. 3 shows serum SOD3 activity, liver Cu concentration, liver CCO activity and ceruloplasmin activity plotted as a function of kidney Cu concentration. These plots are all biphasic, having a linear portion over which little variation occurs in the dependent variable with changes in kidney Cu concentration and a linear portion over which the dependent variable varies greatly with small changes in kidney Cu concentration. Transition points between the two linear portions of the plots were similar for each plot, occurring between kidney Cu concentrations of 18–22 μg Cu/g dry kidney in males and 14–17 μg Cu/g dry kidney in females. The transition points in the plots indicate the point at which the biochemical indices of Cu status begin to rapidly change in relation to kidney Cu concentration. The dietary Cu levels at which the biochemical indices begin to rapidly decline can be calculated from the kidney Cu concentrations at the transition points because kidney Cu and dietary Cu concentrations were strongly correlated in this study over the range from 0 to 3 mg Cu/kg diet in both males and females. The regression equations describing the relationship of kidney Cu to dietary Cu were kidney Cu=7.05+8.47×dietary Cu ($R^2=.65$, $P<.0001$) for males and kidney Cu=8.90+8.63×dietary Cu ($R^2=.54$, $P<.0001$) for females. Dietary Cu contents at the transition points of the biphasic plots calculated using these equations ranged from 1.3 to 1.7 mg Cu/kg in males and from 0.7 to 1.0 mg Cu/kg in females. Kidney Cu concentrations at the transition points in the plots of serum SOD3 activity, liver Cu concentration, ceruloplasmin and liver CCO as functions

Table 4
Estimated kidney Cu concentration and dietary Cu concentration at the transition point of the biphasic plots of serum SOD3 activity, liver Cu concentration, ceruloplasmin activity and liver CCO activity as functions of kidney Cu concentration

	Transition point kidney Cu [μg Cu/g dry weight (95% confidence interval)]		Transition Point Dietary Cu [mg Cu/kg (95% confidence interval)]	
	Males	Females	Males	Females
Serum SOD3	18.1 (17.2–19.1)	17.1 (16.2–18.0)	1.30 (1.10–1.48)	0.95 (0.61–1.19)
Liver Cu	18.6 (17.9–19.3)	16.5 (15.8–17.1)	1.36 (1.16–1.54)	0.88 (0.52–1.13)
Ceruloplasmin	21.6 (19.6–23.6)	17.1 (15.5–18.7)	1.72 (1.54–1.92)	0.95 (0.61–1.19)
Liver CCO	19.3 (16.1–18.4)	14.6 (13.0–16.3)	1.45 (1.26–1.63)	0.66 (0.24–0.94)

of kidney Cu and the estimated dietary Cu concentration at the transition points are shown in Table 4.

4. Discussion

Preliminary experiments with rats fed a nutritionally adequate diet showed that low-molecular-weight substances in serum produced little interference when serum SOD3 activity was assayed at pH 10 with X/XO and acetylated ferricytochrome as superoxide generator and detector, respectively. The X/XO method also was approximately 5 times more sensitive than measuring serum SOD3 activity by monitoring the inhibition of pyragallol autooxidation. We postulated that because of the increased sensitivity and lower interference, serum SOD3 activity could be a sensitive index of Cu status when assayed with the X/XO method.

Of concern is whether serum SOD activity measured in our study is representative of SOD3 activity. We showed that about 60% of the total recoverable serum SOD activity was bound by concanavalin A–Sepharose. This is somewhat low but is in reasonable agreement with normal recoveries of bound SOD3 from conconavalin A–Sepharose columns, which vary between 70% and 90% [19]. However, immunoreactive SOD3, but no SOD1, was detected in both the bound and unbound fractions obtained from the conconavalin A–Sepharose column. Collectively, these findings indicate that the SOD activity in rat serum is due primarily to SOD3.

Several studies have indicated that plasma SOD3 activity is sensitive to Zn status. Reductions of 30–40% in plasma SOD3 activity have been reported in severely Zn-deficient rats [24,25]. It has also been reported that plasma SOD3 is reduced 45% in moderately Zn-deficient female rhesus macaques [25] and that serum SOD3 activity is reduced 10% in women with low Zn intakes [26]. In contrast, our study indicated that serum SOD3 activity was not significantly affected by feeding male rats diet containing 3 mg Zn/kg. The discrepancy between our findings and the reported findings showing that Zn deficiency lowers plasma SOD3 activity in rats most likely reflects differences in the severity of Zn deficiency. In our study, the lowest dietary concentration of Zn was 3 mg/kg, whereas the dietary Zn concentrations in studies showing an effect of Zn deficiency on plasma SOD3 activity were less that 1 mg Zn/kg. In addition, the studies investigating the effect of Zn deficiency on plasma SOD3 activity used the inhibition of pyrogallol autooxidation as the assay method. Although severe Zn deficiency may lower plasma SOD3 activity in rats when measured by monitoring inhibition of pyrogallol autooxidation, our results indicate that moderate Zn deficiency in rats does not affect serum SOD3 activity when assayed by the X/XO method. Furthermore, serum SOD3 was reduced in rats consuming diets containing 0 and 1.5 mg Cu/kg regardless of dietary Zn in our study. This suggests that serum SOD3 activity assayed by the X/XO method is relatively more sensitive to moderate dietary Cu intakes than to moderate Zn intakes in rats.

The second experiment of our study compared the response of serum SOD3 activity assayed by the X/XO method with the response of several more traditional indices of Cu status to graded levels of dietary Cu. In terms of response, serum SOD3 activity was significantly decreased in male rats consuming diets containing 0 and 1 mg Cu/kg and in female rats consuming 0 mg Cu/kg compared with male and females consuming diet containing 6 mg Cu/kg. The decrease in serum SOD3 activity at these dietary Cu levels paralleled the decreases observed for liver Cu concentration, kidney Cu concentration, liver CCO activity and plasma ceruloplasmin activity in males, which began to significantly decrease when dietary Cu concentrations were 1–2.5 mg Cu /kg depending on the index measured. The response of serum SOD3 to dietary Cu in our study also was similar to the response previously reported for red blood cell SOD1 activity, which was shown to decrease in male rats when diets contained less than 3 mg Cu/kg [17]. The change in serum SOD3 activity with dietary Cu also was similar to the change in liver Cu, liver CCO activity and ceruloplasmin activity in females where significant reductions were observed only in rats that consumed diet containing 0 mg Cu/kg. The similarity in the responses of serum SOD3 activity and more traditional biochemical indices of Cu status to dietary Cu content suggests that serum SOD3 activity is a useful index of Cu status in male and female rats.

The response of serum SOD3 activity was more sensitive than the response of hematological parameters and heart size to dietary Cu. Although serum SOD3 reductions were observed with diets containing 0 and 1 mg Cu/ kg in males and 0 mg Cu/kg in females, the lowest dietary concentration of 0 mg Cu/kg produced only a very mild anemia as indicated by a trend toward lower hemoglobin concentrations and hematocrits and elevated red cell distributions. Furthermore, cardiomyopathy was observed only in males consuming diet containing 0 mg Cu/kg. Anemia and cardiomyopathy are commonly observed in Cu deficiency [27,28], and their absence, except for cardiomyopathy in males, suggests that hematological outcomes and cardiac hypertrophy may lag behind changes in biochemical indices of Cu deficiency. In addition, the absence of cardiomyopathy in females consuming a severely Cu-deficient diet indicates that male and female rats differ in their susceptibility to the cardiac consequences of Cu deficiency and as a result, males may develop cardiomyopathy more rapidly than females.

Our findings indicate that serum SOD3 activity is a promising indicator of Cu status because it is sensitive to low dietary intakes and responds in a manner similar to other biochemical indices of Cu status. However, an essential property of any biochemical index of Cu status is its ability to accurately reflect organ Cu reserves. The liver contains a storage compartment for Cu that may be used to

supply Cu to other organs during Cu deficiency [29,30]. Thus, reductions in liver Cu concentration caused by low dietary Cu are a definitive indication of declining Cu status. The linear correlation between serum SOD3 activity and liver Cu concentrations in males and females over the entire dietary range of dietary Cu levels used in this experiment was highly significant and indicates that serum SOD3 activity accurately reflects hepatic Cu reserves in severely and marginally Cu-deficient rats and is as definitive of Cu status as liver Cu concentrations.

Kidney Cu concentration was strongly and positively correlated with dietary Cu concentrations. This observation is consistent with a previous report by Saari [31] showing that kidney copper concentration is a good index for resolving the effects of marginal copper deficiency. The report by Saari also suggested that mathematical regression of a putative Cu-dependent variable against a reliable index of Cu status has potential for discerning the effects of marginal Cu deficiency. However, in the present study plots of serum SOD3 activity as a function of kidney Cu concentration were not linear over the entire range of kidney Cu concentrations. Instead, the plots consisted of two linear portions with a well-defined transition point at 18 μg Cu/g dry kidney in males and 17 μg Cu/g dry kidney in females. This indicates that serum SOD3 activity is not as responsive as kidney Cu concentration to a wide range of dietary Cu intakes. However, the transition points for the plots of serum SOD3 activity as a function of kidney Cu in males and females were similar to the transition points for plots of liver Cu concentration, liver CCO activity and ceruloplasmin activity as functions of kidney Cu. These findings suggest that although kidney Cu responds in a sensitive linear manner to relatively small incremental changes in dietary Cu, kidney Cu must fall below a critical threshold of 15–17 μg Cu/g dry kidney in males and 18–22 μg Cu/g dry kidney in females before serum SOD3 and the other Cu indices begin to decline. The fact that serum SOD3 activity and the other biochemical indices of Cu status exhibit similar critical thresholds for kidney Cu indicates that serum SOD3 activity is as accurate as other indices of Cu status.

The strong, positive linear correlation between kidney Cu concentration and dietary Cu concentration makes it possible to mathematically estimate the dietary Cu concentrations that correspond to the kidney concentrations at the critical transition points in the plots of the various indices of Cu status as a function of kidney Cu. If marginal dietary Cu deficiency is defined as the dietary Cu level below which biochemical indices of Cu status begin to decline relative to a responsive pool of organ Cu, then marginal dietary Cu, within 95% confidence limits, would be 1.1–1.5 mg Cu/kg diet for weanling male rats and 0.6–1.2 mg Cu/kg for weanling female rats based on serum SOD3 activity and kidney Cu concentration.

Our observations indicate that serum SOD3 activity is a biochemical index of Cu status when it is assayed under conditions that increase sensitivity and reduce interferences. However, SOD3 is the most abundant form of SOD in serum [1] and because of its abundance, contributes to most if not all of serum SOD activity. It is well known that the expression of SOD3 is influenced by several factors. Inflammatory cytokines, such as IFN-γ and IL-4, up-regulate SOD3 whereas TNF-α down-regulates the enzyme in vascular smooth muscle cells [32]. The presence of an NF-κB regulatory element in the promoter region of the SOD3 gene indicates that cytokine-induced SOD3 expression is transcriptionally regulated by NF-κB [33]. Oxidants also affect the activity of SOD3 in cultured cells, but the effects are small and most likely caused by cytotoxicity of the oxidants [34]. Thus, potential interferences by inflammation and redox status must be considered when interpretations of Cu status are made based on serum SOD3 activity.

Unique features of rat SOD3 also need to be considered in relating our findings to the suitability of serum SOD3 activity as an indicator of Cu status in humans. In humans, and most other mammals, SOD3 is a tetramer having high affinity for heparin. Heparin Sepharose chromatography divides plasma SOD3 into fractions having no heparin affinity (SOD3-A), weak heparin affinity (SOD3-B) and strong heparin affinity (SOD3-C) [1]. However, rat SOD3 is a dimer that exists only in the A and B forms [22]. As a result of its poor affinity for heparin, rat SOD3 binds the matrix and surfaces of cells with less affinity than SOD3 of other species and achieves higher levels in rat serum compared with other species, including humans, having the C form with high heparin affinity. The lower concentration of SOD3 in human compared with rat serum may decrease the sensitivity of the assay for detecting subtle changes in human Cu status. Mutations also can occur in the heparin-binding domain that accelerate the release of SOD3 from tissue and greatly increase its plasma concentration [35,36]. Another factor that can lead to increased SOD3 levels in serum is proteolytic cleavage of the heparin-binding domain that promotes the clearance of SOD3 from tissue [37,38]. Thus, serum SOD3 activity can be influenced by several factors unrelated to Cu status.

In summary, we found that the assay of serum SOD3 activity in rats at pH 10 with X/XO and acetylated cyt *c* has higher sensitivity to SOD3 activity than the assay utilizing pyragallol, is not susceptible to interferences caused by low-molecular-weight substances and is not highly influenced by moderately low dietary Zn. Furthermore, serum SOD3 activity assayed with X/XO and acetylated cyt *c* parallels the changes of several established biochemical indicators of Cu status in response to dietary Cu intake, is linearly correlated to liver Cu concentrations and is as sensitive to changes in kidney Cu concentrations as other biochemical indices of Cu status. These findings indicate that serum SOD3 activity assayed at pH 10 with X/XO and acetylated cyt *c* is a sensitive biomarker of Cu status. However, it should be recognized that no single index gives an accurate

assessment of Cu status. Potential interferences caused by inflammatory cytokines and low Zn intake require that serum SOD3 activity be used in conjunction with other indices of Cu status, indices of Zn status and biochemical indicators of inflammation to accurately assess Cu status. Although the sensitivities of serum SOD3 and erythrocyte SOD1 to Cu status were not directly compared in the present study, a previous report regarding the response of erythrocyte SOD1 to Cu intake [17] suggests they may be similar in their sensitivity to Cu status. However, assay of serum SOD3 activity is more convenient than the assay of erythrocyte SOD1 activity because it does not require cellular lysis and extraction of hemoglobin. Because the acquisition of serum is relatively noninvasive, the utility of serum SOD3 activity as an index of Cu status in humans needs to be ascertained.

Acknowledgments

The authors thank Kim Michelson, Steve Dufault and Bill Martin for their technical assistance and Denice Schafer for animal care.

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