MAINTENANCE REQUIREMENTS FOR COPPER IN ADULT MALE MICE FED AIN-93M RODENT DIET

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ABSTRACT

Adult male Swiss-Webster mice were fed diets similar to AIN-93M containing 0.8, 1.7, 2.5, 3.6, 4.3, 5.4, and 6.5 mg copper/kg. Significant reductions in serum copper, serum ceruloplasmin amine oxidase activity, and liver copper concentrations were observed in mice fed dietary copper concentrations lower than 2.5 mg/kg. By using nonlinear modeling of serum copper concentrations and serum ceruloplasmin amine oxidase activity, it was estimated that the minimal dietary concentration of copper to maintain maximal levels of these parameters in adult male mice was between 2.5 and 4 mg/kg diet.

KEY WORDS: Copper, Ceruloplasmin, Liver, Superoxide Dismutase, Requirement, Mice

INTRODUCTION

The adult mouse is used extensively in research in immunology, oncology, and toxicology but precise information about the nutritional requirements of this species is lacking. Copper is an essential nutrient for most species of mammals including the mouse, and is required for myriad biochemical and physiological functions. Although the dietary requirement for copper, i.e., the minimal amount of copper in the diet necessary to maintain maximal homeostatic function, has been estimated for rats (1,2), little is known about the copper requirements of the mouse. This study attempts to estimate the minimal copper requirement of adult male mice raised under normal medium-term laboratory procedures, i.e., 12 weeks, in shoe-box cages with multiple tenants, and containing bedding material to collect waste. Recently, the American Institute of Nutrition released a report describing a new purified diet for laboratory rodents (3). The maintenance formulation they prescribe for adult animals, designated AIN-93M, was used in this study.

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MATERIALS and METHODS

Animals and Diet

Fifty-six adult, 5-month-old, male Swiss-Webster mice were purchased from Sasco (Omaha, NE). They were randomly divided into 7 groups of 8 mice each. The mice were kept under normal husbandry conditions, and housed 4 mice to a cage. The cages were the shoe-box type made of plexiglas with stainless-steel tops containing reservoirs for pelleted diet and drinking water. Ground corn cobs (Bed-O'Cob, 3 mm, Anderson's Cob Division, Delphi, IN) were used for bedding material and changed every 2 days. By analysis, the bedding was found to contain 1.6 mg copper/kg. The mice were kept in a temperature (23 ± 0.5°C) and humidity (50 ± 5%) controlled room with a 12-hr light cycle (7:00 AM to 7:00 PM, lights on). Diet and distilled water were supplied continuously throughout the experiment. To identify the mice in each cage, each was marked with an ear-punch. Body weight of individual mice were recorded each week.

The diets were similar to AIN-93M (3) except that they contained variable concentrations of copper. The diets were prepared by our animal diets kitchen and pelleted in our facilities. By analysis, the diets contained 0.8 ± 0.07, 1.7 ± 0.07, 2.5 ± 0.01, 3.6 ± 0.18, 4.3 ± 0.10, 5.4 ± 0.20, and 6.5 ± 0.08 mg copper/kg diet. Each group of mice was fed its respective diet for 12 weeks.

The study was approved by the Animal Use Committee of the USDA, ARS, Grand Forks Human Nutrition Research Center and was in accordance with the guidelines of the National Institutes of Health on the experimental use of laboratory animals (4).

Analytical Methods

At the end of the experiment, each mouse was anesthetized with an intraperitoneal dose (1.5 ml/kg body weight) of a 1:1 ratio of ketamine-HCl:xylazine-HCl solutions (100 and 20 mg/ml, respectively). The abdominal cavity was opened and blood withdrawn from the abdominal aorta by the use of a 25 gauge needle attached to a 1 ml syringe. About 1 ml of blood could be obtained with this procedure before the animal expired. Blood from the needle wound was collected to determine the percent hematocrit.

Liver, kidneys, and heart were removed from each mouse and kept frozen until analyzed for mineral contents and enzyme activity. Serum was collected from the clotted blood and frozen until analyzed for copper and ceruloplasmin activity.

Tissues were analyzed for copper and iron by atomic absorption spectrometry (AAS). Briefly, the tissues were weighed, lyophilized to constant weight, and charred in a muffle furnace at 450°C for 12 hr. Each of the charred samples was suspended in 2 ml aqua regia and heated to dryness on a hot-plate. The samples were returned to the oven and heated at 450°C for another 12 hr. The mineral residue was dissolved in 1 ml aqua regia and diluted appropriately with deionized water. Liver standard reference material (National Institute of Standards and Technology, Gaithersburg, MD) was analyzed with each batch of tissue samples for quality control.

3Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.
All analyzed standards were within the specified range for each mineral. Copper concentration in serum was determined by furnace AAS after diluting the sample 1:5 with deionized water.

Fresh liver samples were analyzed for superoxide dismutase (SOD, EC 1.15.1.1) activity by the methods of Marklund and Marklund (5) and Prohaska et al. (6), and for cytochrome c oxidase (CCO, EC 1.9.3.1) activity by the method of Prohaska and Wells (7). Serum ceruloplasmin amine oxidase (CPAO, EC 1.16.3.1) activity was determined by the methods of Schosinsky et al. (8) and Lehmann et al. (9).

Statistical Analysis

The data for each parameter were analyzed by analysis of variance (ANOVA) and represented in tables as the means of 8 replicates per group with the variance being expressed as the root mean square error (RMSE). RMSE is the square root of the error mean sum of squares term generated by the ANOVA procedures (10), and estimates the average standard deviation from the mean across all groups. For data in graphic form, variances were expressed as the standard error of the individual group mean (SEM).

Models of the form \( y = \alpha (1.0 - e^{-\beta t}) \), where \( y \) was serum copper, serum ceruloplasmin activity, or liver copper and \( t \) was dietary copper in mg/kg were fitted to the data by using nonlinear regression techniques (SAS, SAS Institute Inc., Cary, NC). In this model, \( \alpha \) is an asymptote that \( y \) approaches at rate \( \beta \). Simple linear models of serum copper, serum ceruloplasmin activity, or liver copper versus dietary copper were fitted by linear regression (SAS). The mean square error from each model and the plot of the residuals (residual = predicted value – actual value) versus predicted values were examined to determine whether the linear or nonlinear model provided the best fit (11). The first derivative of the nonlinear model was used to find the concentration of dietary copper at which an increase of one unit resulted in less than one unit increase in the parameter of interest. This point then was used to estimate the minimal concentration of dietary copper required to maximize the particular parameter under the conditions of the experiment.

RESULTS

The mean weight gain for each group during 12 weeks of feeding was approximately 12 g, but was not significantly different among groups (Table 1). None of the possible indicators of copper status listed in Table 1 were significantly affected by this range of dietary copper. These included hematocrits, liver iron concentration, liver CCO activity, heart and kidney copper concentrations.

Indicators of copper status that were significantly affected by dietary copper are shown graphically in Figure 1. As dietary copper increased from 0.8 to 6.5 mg/kg, the concentration of serum copper and the activity of serum CPAO significantly increased (P range 0.05-0.001). We used the first derivative of the nonlinear model to find the concentration of dietary copper at which an increase of one unit resulted in less than one unit increase in serum copper. This point was then used to estimate the minimal concentration of dietary copper required to maintain serum copper at a maximal concentration in adult male mice. Under the conditions of this experiment, this value was approximately 2.5 mg copper/kg diet. By using a similar technique with CPAO activity, the
FIG 1. Effects of dietary copper concentration on serum copper concentration (filled squares) and serum ceruloplasmin amine oxidase (CPAO) activity (open squares). Points represent means ± SEM for 8 replicates per point. A unit of CPAO activity is defined as that volume of serum that oxidizes 1 μmol of o-dianisidine/min. The solid lines in each graph were generated by the equations: Serum copper = 15.19 * \(1 - e^{-1.17(Diet \ copper)}\) and Serum CPAO = 30.55 * \(1 - e^{-0.81(Diet \ copper)}\), respectively. The constants were estimated by using nonlinear modeling of the data. The inset in the lower graph is a linear regression of the means for serum copper and CPAO activity.
estimated minimal dietary concentration of copper to maximize serum CPAO activity was approximately 4 mg copper/kg diet.

There was also a significant increase (P<0.05) in the amount of copper in the livers as dietary copper increased (Figure 2). Liver copper was lower in mice eating diets with 0.8 and 1.7 mg copper/kg than those eating diet with 5.4 and 6.5 mg copper, but no differences occurred among the others. However, the linear model best described these data, which suggests a constant increase in liver copper as dietary copper was increased. Liver SOD activity, on the other hand, was not significantly affected over the range of dietary copper used in this experiment.

**TABLE 1**

Values for Various Physiological Parameters in Adult Male Mice Fed Different Concentrations of Dietary Copper. *

<table>
<thead>
<tr>
<th>Dietary Cu, µg/kg</th>
<th>0.8</th>
<th>1.7</th>
<th>2.5</th>
<th>3.6</th>
<th>4.4</th>
<th>5.4</th>
<th>6.5</th>
<th>RMSE†</th>
</tr>
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<tbody>
<tr>
<td>Body Weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Beginning</td>
<td>26.1</td>
<td>26.1</td>
<td>26.1</td>
<td>26.0</td>
<td>25.8</td>
<td>25.6</td>
<td>25.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Ending</td>
<td>38.4</td>
<td>39.9</td>
<td>39.4</td>
<td>39.9</td>
<td>37.2</td>
<td>37.2</td>
<td>37.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>42.8</td>
<td>41.9</td>
<td>43.0</td>
<td>42.5</td>
<td>42.4</td>
<td>42.3</td>
<td>41.5</td>
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<tr>
<td>Liver</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe, mmol/kg</td>
<td>6.7</td>
<td>5.8</td>
<td>5.2</td>
<td>6.0</td>
<td>6.4</td>
<td>6.0</td>
<td>6.6</td>
<td>1.3</td>
</tr>
<tr>
<td>CCO Activity ‡,</td>
<td>414</td>
<td>391</td>
<td>330</td>
<td>396</td>
<td>461</td>
<td>368</td>
<td>425</td>
<td>170</td>
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<tr>
<td>mU/mg Prot.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Heart</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu, µmol/kg</td>
<td>368</td>
<td>389</td>
<td>374</td>
<td>378</td>
<td>363</td>
<td>389</td>
<td>396</td>
<td>34</td>
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<tr>
<td>Fe, mmol/kg</td>
<td>5.8</td>
<td>6.3</td>
<td>6.3</td>
<td>6.1</td>
<td>6.2</td>
<td>6.4</td>
<td>6.2</td>
<td>0.4</td>
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<tr>
<td>Kidney</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu, µmol/kg</td>
<td>302</td>
<td>305</td>
<td>295</td>
<td>303</td>
<td>296</td>
<td>291</td>
<td>288</td>
<td>30</td>
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<tr>
<td>Fe, mmol/kg</td>
<td>6.8</td>
<td>6.4</td>
<td>5.9</td>
<td>6.1</td>
<td>6.8</td>
<td>6.1</td>
<td>6.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*Values are the mean of 8 replicates per group. †RMSE = root mean square error. ‡One unit of CCO activity is that amount which catalyzes the oxidation of 1 µmol of cytochrome c/min at 30°C.

**DISCUSSION**

To our knowledge, no systematic study to estimate the minimal dietary requirement of copper for adult rodents (mice in particular) has been done. It is generally assumed that either the requirements would be the same as for young growing animals or that the copper stores in adult animals would be sufficient to maintain them for long periods. Recently, there have been studies to estimate copper requirements for young growing rats raised in a totally copper-free environment. Johnson et al. (1) fed weanling male rats purified diets with copper concentrations ranging from 0.2 to 5.4 mg/kg for 5 weeks, and found that numerous parameters used to assess copper status (CPAO activity, plasma copper, SOD activity, etc.) were depressed in rats fed diets with copper concentrations at 3 mg/kg or below. Klevay and Saari (2) fed weanling male rats purified diets with
FIG 2. Effect of dietary copper concentration on liver copper concentration (filled circles) and liver Cu, Zn-SOD activity (open circles). Points represent means ± SEM for 8 replicates per point. One unit of SOD activity is that amount which inhibits pyrogallol autoxidation by 50% at 25°C. Lines through points for each graph were generated by simple linear regression.
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copper concentrations ranging from 0.2 to 5.2 mg/kg for 5 weeks, and found that parameters such as liver copper, heart copper, and serum CPAO activity were depressed in rats fed diets with copper concentrations at 4 mg/kg or below. These two studies suggest that the minimal dietary requirement for copper to maximize numerous parameters used to assess copper status of the young growing rat is at least 4 mg/kg.

Reeves et al. (12) fed weanling male and female mice the AIN-93G purified diet that contained 6 mg copper/kg (3) for up to 17 weeks. They found that the initial weight gain was as good or better than similar mice fed Purina Certified Rodent Diet-5002, a non-purified, cereal-based diet that contains 17 mg copper/kg. By the end of 13 weeks, male but not female mice fed the purified diet weighed significantly more than those fed the non-purified diet. This study does not provide evidence of a minimal requirement for copper, but does suggest that at least 6 mg/kg is enough for adequate weight gain and weight maintenance for up to 17 weeks.

The concentration of copper in serum or plasma, serum CPAO activity, liver copper and iron concentration, liver SOD and CCO activities, and heart copper are often used to assess copper status of animals. In the present study, we used these parameters to assess the dietary requirements of copper for adult male mice. The mice were fed diets with copper concentrations ranging from 0.8 to 6.5. There were no adverse effects on liver SOD or CCO activities, liver iron, or heart copper. However, serum copper and CPAO activity, and liver copper were significantly lower in mice fed diets with the low range of copper. Johnson et al. (1) showed a strong direct relationship between liver copper concentration and liver Cu, Zn-SOD activity in young growing rats. They suggested that this might be used as a functional indicator of copper status. However, in adult male mice, although liver copper significantly increased as dietary copper increased, there was no effect on liver Cu, Zn-SOD activity. Likewise, liver CCO activity is strongly correlated with liver copper concentration in the growing animal (1), but there was no correlation in adult mice. On the other hand, a longer experimental period might have revealed significant changes.

Perhaps the best indicators of copper status in adult mice are serum or plasma copper concentration and/or CPAO activity. To estimate the minimal requirement of copper, we used the model of the form \( y = \alpha (1.0 - e^{\beta t}) \), where \( y \) was serum copper or serum CPAO activity, and \( t \) was dietary copper in mg/kg. The model was fitted to the data by using nonlinear regression techniques. In this model, \( \alpha \) is an asymptote that \( y \) approaches at rate \( \beta \). The first derivative of this model was used to find the concentration of dietary copper at which an increase of one unit resulted in less than one unit of increase in either serum copper concentration or serum CPAO activity. This point then was used to estimate the minimal concentration of dietary copper required to maximize these parameters under the conditions of the experiment. When serum copper was used, the minimal dietary concentration was approximately 2.5 mg/kg. When serum CPAO activity was used, it was approximately 4.0 mg copper/kg diet. Based on these data, the minimal concentration of dietary copper needed to maintain adequate copper status for adult male mice under normal husbandry is approximately 4.0 mg/kg. This compares to 3 to 4 mg/kg for young growing rats (1,2) raised in a copper-free environment.

Because the material used for bedding contained 1.6 mg copper/kg, it could be argued that the mice might obtain some copper from the bedding. Even if the mice consumed as much as 10% of the daily food intake as bedding, which is highly unlikely, they would only take in approximately 0.56 \( \mu \)g copper/day; the amount consumed by those fed the lowest copper diet was 2.8 \( \mu \)g/day. Therefore, the use of this type of bedding material probably would not influence the estimate of copper requirement for adult male mice.
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REFERENCES


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