Development of copper deficiency in neonatal mice

Joseph R. Prohaska

Department of Biochemistry, School of Medicine, University of Minnesota, Duluth, MN, USA

Dietary copper (Cu) deficiency was produced in Swiss albino mice to determine the temporal relationship between depletion of Cu and changes in the cardiovascular and nervous system. Dams were placed on a Cu-deficient diet 4 days after parturition. Half the dams were provided with deionized water and their offspring are referred to as Cu-deficient (−Cu). Half the dams were given cupric sulfate in their drinking water (20 μg Cu/mL) and their offspring are referred to as Cu-adequate (+Cu). At 6 weeks of age a sample of the −Cu mice were repleted with $CuSO_4$. Mice were sampled 1 day after birth and at weekly intervals for 7 weeks. Both +Cu and −Cu mice grew at the same rate; birth weight increased 16-fold at 6 weeks of age. Liver Cu more than doubled between 1 and 7 days of age. At 2 weeks of age −Cu mice were anemic (lower hematocrit and hemoglobin) and had lower liver Cu and plasma ceruloplasmin activity compared to +Cu mice. Liver Fe was not elevated in −Cu mice until 2 weeks after anemia developed. At weaning first signs of altered catecholamine metabolism included elevation of dopamine in both heart and spleen. Norepinephrine concentrations and content, in contrast, were not both lowered in −Cu mice until 5 weeks of age. Heart weight was first elevated in −Cu mice at 6 weeks of age and relative weight (mg/g body wt) at 4 weeks of age. Liver Cu concentration was lower in 1-week repleted mice than in +Cu mice. Anemia preceded the development of cardiac hypertrophy and altered catecholamine levels in −Cu mice.

Keywords: copper deficiency; mice; development; norepinephrine; dopamine

Introduction

Copper is an essential trace metal that exhibits its biological properties by serving as a cofactor for a number of important cuproenzymes. When copper is limiting these cuproenzymes may become rate limiting and lead to metabolic and pathologic changes. In addition, a number of other enzymes change in activity when copper is limiting.[1] These indirect changes may also be involved in explaining the complex pathophysiology that accompanies copper deficiency.

Like many essential nutrients, copper is most critical during neonatal development.[2,3] During the later stages of gestation and early lactation copper deficiency is likely to occur in offspring that do not receive enough copper from their mothers. The precise temporal sequence of copper transfer is species specific.[2]

A murine model has been investigated to elucidate the copper-dependent mechanisms responsbile for development and maintenance of the cardiovascular and immune system. Swiss albino mice nursed by dams fed a copper-deficient diet from parturition and then subsequently weaned to this diet exhibit profound changes to the immune system.[4] At 6 weeks of age these mice were smaller and more anemic when compared to mice in which initiation of copper-deficient treatment was delayed by 1 week.[4] This illustrates the critical nature of copper in neonatal development. In two recent studies with similar mice the dietary treatment was initiated 4 days after birth. The 6-week-old-copper deficient offspring were anemic and had enlarged hearts and lower norepinephrine compared to copper-adequate controls.[5] In addition, the copper-deficient mice had variable responses to changes in organ copper content and catecholamine pool size.[6]

The classical work of Goodman et al.[7] emphasized that the cardiac hypertrophy of copper-deficient rats preceded the development of anemia suggesting that the mitochondrial hypertrophy observed could not be attributed entirely to increased workload secondary to

Address reprint requests to Joseph R. Prohaska, Department of Biochemistry, University of Minnesota, Duluth, MN 55812, USA.
Supported by PHS Grant HD 20975 and Minnesota Medical Foundation Grant DMRF-30-84.
Received December 27, 1989; accepted March 9, 1990.

anemia. However, the documentation of hypertrophy was 7 weeks after initiation of treatment. At that time copper-deficient rats were mildly anemic (hemoglobin 12.3 g/100 mL compared to a control average of 15.4).[7] Thus, the purpose of the present study was to determine the sequence of events in neonatal copper deficiency that lead to changes in the cardiovascular system. A second purpose for this study was to determine the temporal nature of altered catecholamine metabolism in this experimental paradigm because of the possible connection of altered norepinephrine metabolism and cardiac hypertrophy.[8]

Methods and materials

Animal care and diets

Swiss albino mice, Hsd: (ND/4) (S)BR, purchased commercially (Harlan Sprague–Dawley, Indianapolis, IN, USA), were mated to establish a breeding colony, and 2 days following parturition, litter size was adjusted to 8 pups.

Four days after parturition mouse dams were divided into two dietary treatments, copper-deficient (−Cu) and copper-adequate (+Cu), that consisted of feeding a copper-deficient purified diet (Teklad Laboratories, Madison, WI, USA) and either low-copper drinking water or copper-supplemented drinking water, respectively. The purified diet was similar to the AIN-76A diet[9,10] 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; and AIN-76A vitamin mix, 10. Cupric carbonate was omitted from the AIN-76 mineral mix. By analysis, the purified diet contained 0.45 mg copper/kg and 45 mg iron/kg. Offspring and dams on the −Cu treatment drank deionized water containing 0.2 ng Cu/L by analysis, whereas +Cu treatment groups drank water that contained 20 mg Cu/L by adding copper to the drinking water as $CuSO_4$. Diet and drinking water were available ad libitum.

Male mice were weaned when 3 weeks old and placed in stainless steel cages (4 mice per cage) and were maintained on the same treatment as their respective dams for an additional 4 weeks. When 6 weeks of age a sample of the −Cu mice was repleted with the $CuSO_4$ drinking water for 1 week (group R mice). All animals were maintained at 24°C with 55% relative humidity on a 12-h light cycle (0700–1900 h).

Sample collection

Mice were sampled from several litters when they were 1 day old and at weekly intervals thereafter until 7 weeks of age. From age 1 week on only male offspring were analyzed to compare with previous studies with 6-week-old male mice.[5,6]

Mice were killed by decapitation under light ether anesthesia and blood samples were drawn into heparinized microhematocrit tubes. A 5-μL aliquot of blood was also taken for hemoglobin analysis. Livers, hearts, and spleens were removed, weighed, and processed for metal analysis or frozen in liquid nitrogen. Organs for catecholamine analysis were stored at −70°C for no longer than 1 week prior to analysis.

Biochemical analyses

Hemoglobin was determined spectrophotometrically as metcyanhemoglobin.[11] Plasma was obtained following centrifugation of the microhematocrit tube and used to measure the activity of the cuproprotein ceruloplasmin by following oxidation of *O*-dianisidine.[11]

Livers, or pools of livers for 1-day mouse samples, and 1-g portions 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 0.1N HNO_3. Samples were then analyzed for total copper and iron by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT, USA).

Catecholamine analysis

Hearts and spleens were processed for catecholamine analysis using a protocol described in detail previously.[5] Binding to and elution from alumina were carried out at room temperature; samples were kept on ice until fractionated by HPLC. The extracted catecholamines were separated by reverse-phase ion-pair HPLC with electrochemical detection.[5] Pooling of hearts ($N = 3$) was necessary for the 1-day-old mice. Spleens were not analyzed for these mice due to small sample size.

Statistical analysis

Data were obtained from a minimum of 3 litters for each treatment group and age. Mean comparisons between the two treatment groups for a given age were made by utilization of Student's unpaired t test, $\alpha = 0.01$. For the 7-week-old mice the three treatment groups (+Cu, −Cu, and R) were analyzed using one-way ANOVA; mean comparisons were tested using the Scheffe F test, $\alpha = 0.05$. All data were analyzed using a Macintosh personal computer and statistical software (Statview 512+, Brain Power, Calabasas, CA, USA).

Results

Regardless of dietary treatment, growth of offspring between birth and 7 weeks of age was good and no treatment differences were noted (*Figure 1*). However, signs of Cu deficiency were detected very soon after placing dams on the Cu-deficient treatment. By age 2 weeks (10 days after dams were placed on treatment) there was a significant reduction in plasma ceruloplasmin activity in −Cu compared to +Cu pups (*Figure 1*). From age 3 weeks onward −Cu mice had no detectable enzyme activity. There was a rapid fourfold rise in plasma ceruloplasmin activity in +Cu mice

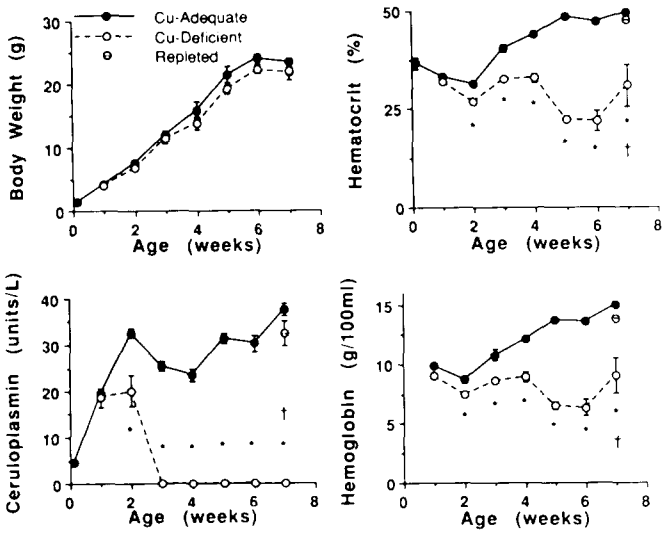

Figure 1 Body weight, hematocrit, plasma ceruloplasmin activity, and hemoglobin levels of neonatal male Swiss albino mice. Points with error bars represent the means ± SEM; the number of mice is given in *Table 1*. At a given age means were compared by Student's *t* test (*$p < .01$) or at 7 weeks by ANOVA and the Scheffe *F* test ($p < .05$, *Cu-adequate vs. Cu-deficient, †repleted vs. Cu-deficient).

between 1 and 7 days of age. By 2 weeks of age mouse plasma ceruloplasmin activity had reached adult levels (*Figure 1*).

The early signs of Cu deficiency were also reflected in the pathophysiological expression of anemia, since both hematocrit and hemoglobin were lower in −Cu mice compared to +Cu mice by 2 weeks of age (*Figure 1*). Hematological differences between −Cu and +Cu mice became more pronounced in older pups as both hematocrit and hemoglobin rose in the +Cu mice with age and fell in −Cu mice. Repletion with $CuSO_4$ in the drinking water of 6-week-old −Cu mice resulted in mice (group R) with average ceruloplasmin activities, hemoglobin, and hematocrit levels not different than +Cu mice but higher than −Cu mice (*Figure 1*).

Liver weight, both absolute and relative, increased with age in mice. There was a major rise in relative weight between age 2 and 3 weeks in both +Cu and −Cu mice, after which time relative liver weight was constant (*Figure 2*). Relative liver weight of 6- and 7-week-old −Cu mice was higher than +Cu mice. Total liver Cu more than doubled between 1 and 7 days of age and remained stable for the next week in +Cu mice, but fell precipitously in −Cu mice (*Figure 2*). This marked accumulation of liver Cu in +Cu mice resulted in a constant concentration of Cu because liver size was increasing (*Table 1, Figure 2*). In +Cu mice, total liver Cu fell between ages 2 and 3 weeks but was still higher compared to −Cu mice. Total liver Cu rose in +Cu mice during postweaning, whereas the concentration tended to stabilize (*Table 1, Figure 2*). Liver Cu content and liver Cu concentration of −Cu mice was lower than that of +Cu mice from age 2 weeks onward. Following the 1 week repletion period, Cu concentration in livers of group R mice were higher than −Cu but lower than +Cu values (*Table 1*). Liver Fe was also measured and showed that +Cu mice began to accumulate liver Fe after weaning (*Figure 2*). At age 5 weeks (3 weeks after anemia had been detected) there was a noticeable accumulation of Fe in livers of −Cu mice compared to +Cu mice. The elevated liver Fe was not eliminated totally following 1 week of Cu repletion. The concentration of Fe in liver was higher in −Cu mice compared to +Cu mice from age 3 weeks onward (*Table 1*). The high concentration of liver Fe measured 1 day after birth fell postnatally and gradually returned to a similar level by 7 weeks of age.

Heart weight increased more than 10-fold during the 7-week study period. At ages 6 and 7 weeks the hearts of −Cu mice were heavier than those of +Cu mice (*Figure 3*). An elevation in relative heart weight of −Cu mice was detected earlier at 4 weeks of age. Cardiac norepinephrine concentration and total content were lower in −Cu mice compared to +Cu mice starting at ages 3 and 5 weeks, respectively (*Figure 3, Table 1*). Dopamine concentration and content, in contrast, were both higher in hearts of −Cu compared to +Cu mice by age 3 weeks. In +Cu mice, total heart norepinephrine and dopamine increased more than 50-fold and 20-fold, respectively, from age 1 day to 7 weeks (*Figure 3*). Cardiac norepinephrine and dopamine levels of group R mice were not different from +Cu mice but were altered compared to −Cu mice (*Figure 3, Table 1*).

Spleen weight increased between 1 and 7 weeks of age approximately threefold. The relative spleen weight dropped between ages 1 and 5 weeks and then appeared constant. Cu-deficient treatment elevated both absolute and relative spleen weight at 7 weeks of age (*Figure 4*). Norepinephrine concentration and con-

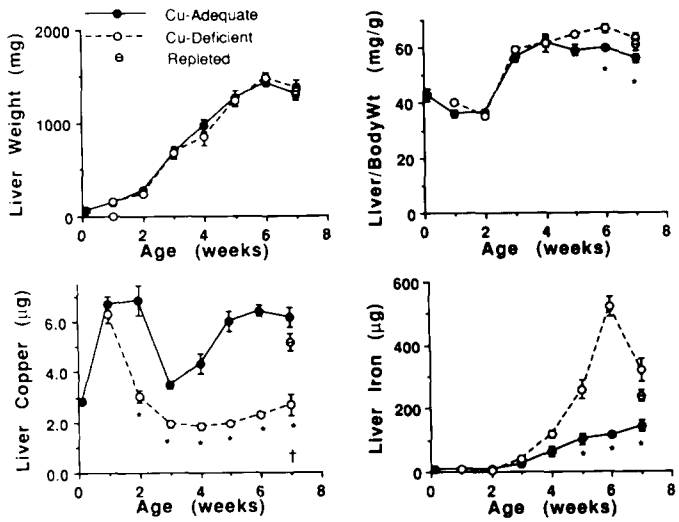

Figure 2 Liver weight and metal content of neonatal male Swiss albino mice. Points with error bars represent the means ± SEM; the number of mice is given in *Table 1*. At a given age means were compared by Student's *t* test (*$p < .01$) or at 7 weeks by ANOVA and the Scheffe *F* test ($p < .05$, *Cu-adequate vs. Cu-deficient, †repleted vs. Cu-deficient).

Table 1 Concentrations of copper, iron, and catecholamines during development of copper deficiency in neonatal Swiss albino mice[a]

Parameter	Diet	Age[b] (weeks) 0.14	1	2	3	4	5	6	7
No. mice	+Cu	3	8	8	8	4	4	4	9
	−Cu		8	15	10	4	5	5	7
	R								5
Liver copper (μg/g)	+Cu	46.5 ± 5.71	44.2 ± 1.45	25.0 ± 2.13	5.34 ± 0.52	4.42 ± 0.14	4.74 ± 0.06	4.50 ± 0.16	4.70 ± 0.10
	−Cu		38.3 ± 1.91	12.9 ± 1.12*	2.90 ± 0.12*	2.18 ± 0.19*	1.58 ± 0.06*	1.53 ± 0.07*	1.94 ± 0.28*†
	R								3.86 ± 0.15§
Liver iron (μg/g)	+Cu	117 ± 20.8	55.4 ± 3.56	26.8 ± 0.92	32.3 ± 1.12	61.3 ± 12.0	80.6 ± 13.9	80.9 ± 2.5	110 ± 14.2
	−Cu		47.4 ± 2.39	23.6 ± 0.88	53.9 ± 3.47*	139 ± 9.52*	214 ± 30.9*	344 ± 5.97*	235 ± 23.0*
	R								180 ± 16.2
Heart norepinephrine (ng/g)	+Cu	223 ± 58.6	207 ± 25.3	435 ± 20.0	673 ± 25.4	771 ± 43.7	795 ± 41.4	903 ± 67.7	862 ± 33.2
	−Cu		259 ± 14.4	413 ± 29.0	510 ± 29.3*	515 ± 17.6*	328 ± 46.1*	475 ± 73.5*	573 ± 80.7*†
	R								820 ± 39.3
Heart dopamine (ng/g)	+Cu	13.6 ± 9.45	5.06 ± 1.42	8.45 ± 1.71	16.2 ± 2.14	14.6 ± 1.79	16.8 ± 1.27	22.7 ± 3.14	23.6 ± 0.72
	−Cu		5.51 ± 0.74	17.0 ± 4.37	29.7 ± 3.01*	87.0 ± 18.8*	156 ± 17.7*	119 ± 26.0*	73.0 ± 14.8*†
	R								20.5 ± 2.63
Spleen norepinephrine (ng/g)	+Cu	ND	74.9 ± 7.14	206 ± 22.4	281 ± 22.2	287 ± 16.7	474 ± 49.6	598 ± 11.0	606 ± 48.8
	−Cu		72.5 ± 4.56	233 ± 29.7	219 ± 19.0	267 ± 35.8	217 ± 12.0*	354 ± 15.5*	377 ± 87.4
	R								487 ± 49.8
Spleen dopamine (ng/g)	+Cu	ND	4.45 ± 1.23	9.01 ± 2.07	7.19 ± 1.13	14.2 ± 5.08	16.4 ± 2.10	24.2 ± 2.78	25.8 ± 2.54
	−Cu		4.38 ± 0.51	10.7 ± 1.70	13.0 ± 1.92*	58.7 ± 11.9*	131 ± 18.7*	131 ± 19.4*	66.7 ± 21.9
	R								21.7 ± 3.45

[a] Dietary copper deficiency was initiated when pups were 4 days of age; offspring (−Cu) were compared to those nursed by dams given adequate copper (+Cu). Values are means ± SEM for male Swiss albino mice ages 1 day–7 weeks. For a given age and treatment a minimum of three litters was sampled. Means (+Cu vs. −Cu) were compared by Student's *t* test; *$p < .01$, for ages 1–6 weeks. A sample of the −Cu mice were switched to the +Cu treatment between 6 and 7 weeks of age (R). Data from these three groups were analyzed by one-way ANOVA and means were compared by the Scheffe *F* test; $p < .05$ (*+Cu vs. −Cu, † R vs. −Cu, § R vs. +Cu). ND, not determined.
[b] Values are means ± SEM for 3 litters. Each litter mean was obtained by pooling organs from 3–5 mice.

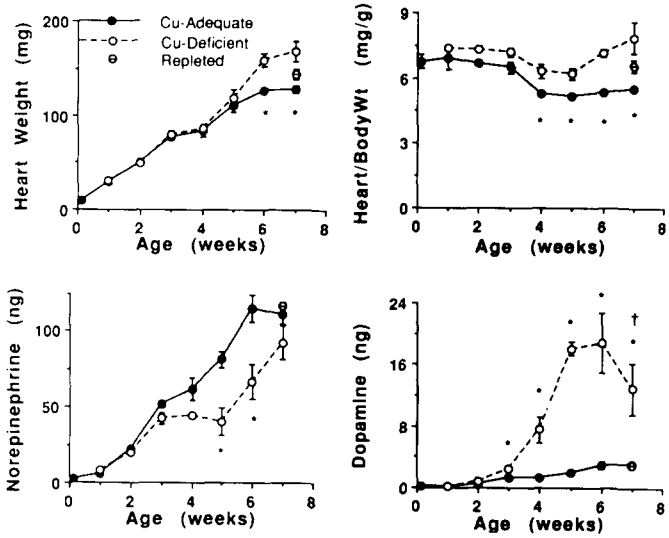

Figure 3 Heart weight and catecholamine content of neoanatal male Swiss albino mice. Points with error bars represent the means ± SEM; the number of mice is given in *Table 1*. At a given age means were compared by Student's *t* test (*$p < .01$) or at 7 weeks by ANOVA and the Scheffe *F* test ($p < 0.05$, *Cu-adequate vs. Cu-deficient, †repleted vs. Cu-deficient).

tent was lower in −Cu compared to +Cu mice starting at ages 5 and 4 weeks, respectively. Spleen dopamine concentration and content was higher in −Cu mice than +Cu starting at 3 weeks of age (*Figure 4, Table 1*).

Discussion

During the neonatal period there are some profound changes in Cu metabolism in the Swiss albino mice. Liver Cu accumulated during the early suckling period and then declined during the last week of lactation. In rats a similar pattern has been noted except that the drop occurs in the week following weaning.[12] Rats, like mice in the current experiments, exhibit low ceruloplasmin activity at birth and demonstrate a rapid rise in activity in the suckling period to adult levels.[12]

The source of the pup liver Cu accretion during early lactation is likely dam's milk. It is known for rats that early milk has higher Cu levels than later milk.[13] This extra Cu is most likely accumulating in intestine for subsequent transfer to liver.[14] The fall in liver Cu concentration with age that was observed in Swiss albino mice is similar to that reported earlier by Keen and Hurley.[15] The concentrations of liver Cu (μg/g wet wt) in neonates were somewhat higher in the current studies than those reported previously.[15] The marked increase in liver Cu during the first postnatal week of mice explains the variable response of mice, observed previously, when onset of dietary Cu deficiency was altered during this period.[4]

Iron concentration in mouse liver from birth to 7 weeks of age follows a bell-shaped pattern. This pattern was observed previously for hybrid mice.[15] In contrast to liver Cu content, liver Fe content began to rise only after weaning. The accumulation of hepatic

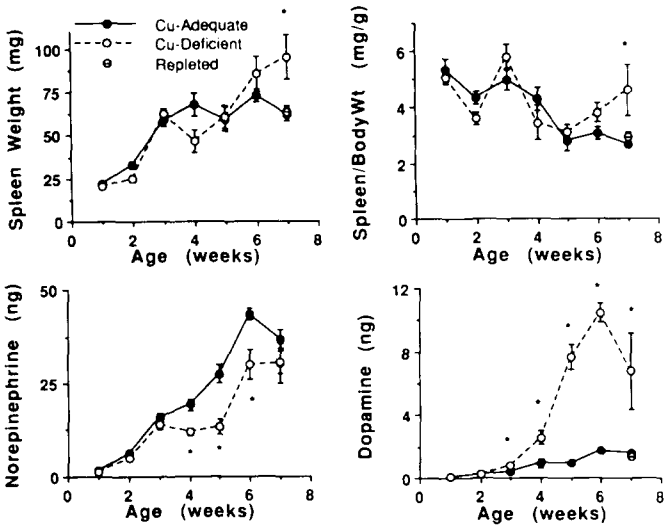

Figure 4 Spleen weight and catecholamine content of neonatal male Swiss albino mice. Points with error bars represent the means ± SEM; the number of mice is given in *Table 1*. At a given age means were compared by Student's *t* test (*$p < .01$) or at 7 weeks by ANOVA and the Scheffe *F* test ($p < .05$, *Cu-adequate vs. Cu-deficient).

Fe in Cu-deficient mice was observed postweaning when anemia became more evident.

The induction of Cu deficiency in the chosen experimental paradigm was evident by 10 days after treatment was started. Although the 2-week-old −Cu mice were somewhat variable in their expression of Cu deficiency, it was clear that the average mouse was already anemic at this age. Later the expression of cardiac hypertrophy occurred. Therefore, anemia preceded hypertrophy in this model. Changes in catecholamine levels were occurring at approximately the same time as changes in heart weight. It is difficult to determine whether a relationship exists between the onset of hypertrophy and alteration in norepinephrine metabolism. Some forms of cardiac hypertrophy appear to be related to norepinephrine outflow.[8] Perhaps altered norepinephrine metabolism has something to do with the cardiac enlargement of Cu-deficient mice.[5] The elevation in dopamine in heart and spleen of young −Cu mice was detected before a reduction in norepinephrine. Previously, it was shown that certain organs of 6-week-old −Cu mice had elevated dopamine but normal norepinephrine levels.[6] Measurement of selected dopamine pools may be a sensitive indicator of Cu status.

Development of enlarged liver and spleen weights to body weights in the −Cu mice observed in the later stages of the current experiments were noted previously.[4] The basis for these organ weight changes is not known.

Restriction of dietary Cu during lactation in mice leads to rapid signs of Cu deficiency in pups. Most of these signs were reversible with 1 week of Cu supplementation. It was interesting to observe, however, that liver Cu concentration remained below control levels despite an intake of more than 500 μg of Cu during the 1 week of supplementation. Functionally, however, the repleted mice did appear similar to control mice. The 1-week supplementation period was sufficient to correct the anemia, eliminate catecholamine imbalances, and partially reduce the enlarged heart.

Acknowledgment

The skillful technical assistance of William R. Bailey is appreciated.

References

1 Prohaska, J.R. (1988). Biochemical functions of copper in animals. In *Essential and Toxic Trace Elements in Human Health and Disease* (A.S. Prasad, ed.), pp. 105–124, Liss, New York
2 Davis, G.K. and Mertz, W. (1987). Copper. In *Trace Elements in Human and Animal Nutrition* (W. Mertz, ed.), pp. 301–364, Academic, New York
3 Danks, D.M. (1988). Copper deficiency in humans. *Annu. Rev. Nutr.* **8**, 235–257
4 Prohaska, J.R. and Lukasewycz, O.A. (1989). Copper deficiency during perinatal development: effects on the immune response of mice. *J. Nutr.* **119**, 922–931
5 Gross, A.M. and Prohaska, J.R. (1990). Copper-deficient mice have higher cardiac norepinephrine turnover. *J. Nutr.* **120**, 88–96
6 Prohaska, J.R., Bailey, W.R., Gross, A.M., and Korte, J.J. (1990). Effect of dietary copper deficiency on distribution of dopamine and norepinephrine in mice and rats. *J. Nutr. Biochem.* **1**, 149–154
7 Goodman, J.R., Warshaw, J.B., and Dallman, P.R. (1970). Cardiac hypertrophy in rats with iron and copper deficiency: quantitative contribution of mitochondrial enlargement. *Pediatr. Res.* **4**, 244–256
8 Östman-Smith, I. (1981). Cardiac sympathetic nerves as the final common pathway in the induction of adaptive cardiac hypertrophy. *Clin. Sci.* **61**, 265–272
9 American Institute of Nutrition. (1977). Report of the AIN Ad Hoc Committee on standards for nutritional studies. *J. Nutr.* **107**, 1340–1348
10 American Institute of Nutrition. (1980). Second report of the AIN Ad Hoc Committee on standards for nutritional studies. *J. Nutr.* **110**, 1726
11 Prohaska, J.R. (1983). Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. J. Nutr. **113**, 2048–2058
12 Terao, T. and Owen, C.A., Jr. (1977). Copper metabolism in pregnant and postpartum rat pups. *Am. J. Physiol.* **232**, E172–E179
13 Keen, C.L., Lönnerdal, B., Clegg, M., and Hurley, L.S. (1981). Developmental changes in composition of rat milk: trace elements, minerals, protein, carbohydrate and fat. *J. Nutr.* **111**, 226–230
14 Mason, R., Brady, F.O., and Webb, M. (1981). Metabolism of zinc and copper in the neonate: accumulation of Cu in the gastrointestinal tract of the newborn rat. *Br. J. Nutr.* **45**, 391–399
15 Keen, C.L. and Hurley, L.S. (1980). Developmental changes in concentrations of iron, copper, and zinc in mouse tissues. *Mech. Ageing Dev.* **13**, 161–176