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Effect of Dietary or Genetic Copper Deficiency on Brain Catecholamines, Trace Metals and Enzymes in Mice and Rats[1,2]

JOSEPH R. PROHASKA AND TIMOTHY L. SMITH

Department of Biochemistry, School of Medicine, University of Minnesota, Duluth, Duluth, MN 55812

ABSTRACT Previous studies by others indicated that alterations in brain catecholamines were different for perinatal copper deficiency produced by diet in rats and that resulting from a genetic mutation on the X-chromosome, Menkes' syndrome in humans and brindled mice. Thus, copper deficiency was studied in a model in which dietary and genetic deficiency (brindled mice) were compared in two strains of the same species, C57BL and C3H/HeJ mice. Dietary copper deficiency was also produced in rats for comparison. In brain, both dietary and genetic copper deficiency resulted in impaired growth, low brain copper levels, greatly decreased norepinephrine concentrations but normal dopamine levels. The activity of brain cytochrome oxidase was greatly depressed following both dietary and genetic copper deficiency, suggesting a functional deficit of copper. However, the activity of another cuproenzyme, dopamine-β-hydroxylase, was significantly elevated in deficient animals. The elevation was observed when either copper or *N*-ethylmaleimide was added to inactivate an endogenous inhibitor. The cause of low brain norepinephrine remains unknown; however, depressed brain norepinephrine may be partly responsible for functional changes in the deficient animals, such as hypomyelination, since the activity of the myelin protein, 2',3'-cyclic nucleotide 3'-phosphodiesterase, was lower in the most deficient animals. J. Nutr. *112:* 1706–1717, 1982.

INDEXING KEY WORDS copper deficiency · brindled mice · catecholamines · dopamine-β-hydroxylase

Many nutritional factors influence the homeostasis of the central nervous system, including catecholamine metabolism (1, 2). Synthesis of the catecholamines, dopamine and norepinephrine, two brain neurotransmitters, depends on adequate levels of tyrosine, their amino acid precursor (3). Vitamins and trace metals also influence catecholamine metabolism through their cofactor roles in enzymes. Iron is most likely required by tyrosine hydroxylase, the first and rate-limiting enzyme in synthesis of dopamine and norepinephrine. The L-dihydroxyphenylalanine (L-dopa) that is formed is then decarboxylated to dopamine by a pyridoxal phosphate–dependent enzyme. In noradrenergic neurons, the dopamine is further converted to norepinephrine by the cuproenzyme, dopamine-β-hydroxylase (DBH). This step also requires ascorbate. Catabolism of dopamine and norepinephrine is initiated by monoamine oxidase, a flavoprotein, or catechol-*O*-methyl transferase, which requires magnesium. Thus, dietary protein (tyrosine), pyridoxine, riboflavin, ascorbate, iron, copper, and magnesium may influence catecholamine metabolism in brain and other tissues.

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Copper deprivation has the most pronounced influence of these nutrients on brain catecholamines. Dietary copper deficiency results in large decreases in the concentrations of norepinephrine in rat (4–7) and lamb (8) brain. Dopamine levels have also been reported to be low in copper-deficient brain (6–8). The decrease in brain norepinephrine may be due to a functional deficit in the cuproenzyme, DBH, although this has not been directly demonstrated. The explanation for decreased dopamine is less clear.

Genetic factors may also influence catecholamine metabolism. A mutation affecting both copper and catecholamine metabolism has been extensively studied by Hunt and coworkers (9–12). They have investigated the X-linked mottled mouse mutants, which are homologous to human congenital copper deficiency, Menkes' syndrome (11). One of the mouse mutants, brindled, has been studied most extensively. The hemizygous males ($Mo^{br/y}$) die at about 2 weeks of age. Their brains are characterized by aberrant ultrastructural features (13), low copper levels (11) and greatly reduced norepinephrine concentrations (9–11), analogous to the copper-deficient rat (4, 5). However, brindled mice, in contrast, have normal dopamine levels in brain (9, 10). It is not known whether this contradiction is due to the mutation or a species difference.

Hunt suggested that the low brain norepinephrine was due to a reduction in DBH (10). However, when estimated in vitro (11), the activity of the enzyme was higher in the brain preparations from brindled mice. This paradox may be explained by the fact that Hunt added exogenous copper to the assay media in his experiments (11) to inactivate the known endogenous inhibitor of DBH. The added copper could have activated apoenzyme.

To investigate further the role of copper in brain catecholamine metabolism, we have produced dietary copper deficiency in two strains of mice. Brindled mice were studied in the same strains. This model was used previously to compare dietary and genetic copper deficiency in regard to another enigma, the lack of anemia in brindled mice and subjects with Menkes' syndrome (14). The present study also utilized albino rats for comparison. Brain levels of catecholamines, copper, iron and specific enzymes were determined.

MATERIALS AND METHODS

Animal care and diets. The experimental model was similar to previous work (14). Hemizygous brindled males ($Mo^{br/y}$) were obtained from matings of heterozygous females ($Mo^{br/+}$) with normal males ($Mo^{+/y}$). Two strains of mice were used, C3H/HeJ (Jackson Laboratories, Bar Harbor, ME) and C57BL (kindly provided by Douglas Grahn, Argonne National Laboratory). These genetic breeding units were given tap water to drink and fed a nonpurified diet (mouse chow, Ralston Purina Co., St. Louis, MO) containing 12–14 ppm of copper by analysis. Dietary copper deficiency was produced by feeding a purified diet low in copper, modified AIN-76A (Teklad Laboratories, Madison, WI), formulated to omit cupric carbonate from the salt mix.[3] This diet averages 0.5 ppm copper and 45 ppm iron by analysis. The diet was fed beginning with the putative gestational period to normal females ($Mo^{+/+}$) and males of both mouse strains. Half the breeding units and subsequent dams were given supplemental copper in their drinking water as cupric sulfate (20 ppm copper), and their offspring served as controls (+Cu). The other dams received deionized water to drink, and their offspring are referred to as copper-deficient (−Cu). Similar studies have been conducted previously (14).

Dietary copper deficiency has also produced in offspring of albino rats (Sprague-Dawley, Madison, WI) by feeding sperm-positive females the purified diet and deionized water (−Cu) during gestation and lactation. Copper-supplemented (+Cu) dams were included as in the mouse studies. Ten dams, five +Cu and five −Cu, and their subsequent litters were studied; the experiment was repeated with ten additional dams. Off-

[3] The composition and efficacy of the purified diet is described in detail elsewhere (20). This modified AIN-76A diet differs from the original AIN-76 diet (20) in that $CuCO_3$ was omitted from the AIN-76 salt mix, the vitamin K content was increased by including menadione sodium bisulfite (0.15 g/kg) in the AIN-76A vitamin mix, and ethoxyquin was added (0.001% of the diet). The major components of the diet are sucrose (50%), casein (20%), cornstarch (15%), corn oil (5%), cellulose (5%), mineral mix (3.5%) and vitamin mix (1%).

spring data from both experiments have been pooled. Some rat offspring were transferred to stainless-steel cages when 3 weeks old and were continued on their respective treatments. Food and water were provided ad libitum and no apparent differences in food intake were noted.

Chemical analyses. Total copper and iron were determined on fresh tissues by flame atomic absorption spectroscopy (Perkin-Elmer model 2380, Perkin-Elmer Corp., Norwalk, CT) after wet-digestion in nitric acid. Tissue protein was estimated by a modification of the Lowry procedure as described previously (14). Blood hemoglobin levels were measured as cyanmethemoglobin (14). Whole brain[4] dopamine and norepinephrine levels were quantified by a modification of the method of Felice et al. (15), which employs isolation of the amines on alumina and subsequent analysis by reverse-phase ion-pair liquid chromatography (HPLC) with electrochemical detection. Fresh brains or brains that had been frozen in liquid nitrogen and stored at −75° were homogenized (Tissumizer, Tekmar Co., Cincinnati, OH) in cold 0.05 *N* $HClO_4$ containing dihydroxybenzylamine as internal standard (Aldrich Chemicals, Milwaukee, WI). The supernate following centrifugation (Model J21C, JA 20 rotor, Beckman Instruments, Palo Alto, CA) at 12,000 × *g* for 15 minutes was processed according to Felice et al., except that 0.1 *N* $HClO_4$ was required to elute the amines from the alumina. Chromatography was carried out at 45° by HPLC (Altex pump model 110A, Altex Scientific Inc., Berkeley, CA; Rheodyne injector model 7125, Rheodyne Inc., Cotati, CA) on a 4.6 × 250 mm Lichrosorb RP-18 10 μm column (Hibar II, American Scientific Products, McGaw Park, IL) equilibrated with a mobile phase consisting of 5% methanol and 95% 0.1 M potassium phosphate, 0.1 mM EDTA, 0.4 mM sodium octyl sulfonate (Regis Chemical Co., Morton Grove, IL) (pH 3.0). Catecholamines were measured by using an electrochemical detector (Bioanalytical Systems, Inc., West Lafayette, IN) consisting of a carbon paste electrode set at 0.72 volts versus the Ag/AgCl reference electrode. Peaks were integrated (Hewlett-Packard model 3385, Hewlett-Packard Co., Palo Alto, CA), and dopamine and norepinephrine were quantified by using the internal standard method (15). Excellent separation of the amines is obtained by these procedures (16).

Enzyme assays. Several cuproproteins were estimated by measuring their catalytic activities. Serum ceruloplasmin (EC 1.16.3.1) was measured by using *o*-dianisidine as substrate as described previously (14). Brain cytochrome oxidase (EC 1.9.3.1) was determined on fresh homogenates by following loss of ferrocytochrome *c* at 550 nm (Beckman DU-2, Beckman Instruments; Gilford 2000 spectrophotometer, Gilford Instrument Laboratories, Oberlin, OH) as described previously (4).

Dopamine-β-hydroxylase (EC 1.14.17.1) (DBH) was measured spectrophotometrically (Beckman model DU-8) by a modification of the dual-wavelength method of Kato et al. (17). Mouse brains were removed from animals, and the cerebrum and cerebellum were discarded. The remainder was homogenized in 24 volumes of 5 mM potassium phosphate (pH 7.0) containing 0.2% Triton X-100. The mixture was centrifuged (6000 × *g* for 10 minutes), and 0.5 ml of the resulting supernate was used to determine enzyme activity. The final volume of the reaction mixture was adjusted to 1.0 ml and contained the following components at their final concentrations: sodium acetate (0.1 M, pH 5.5); sodium fumarate (22 mM); pargyline (2 mM); L-ascorbate (12 mM); *N*-ethylmaleimide (NEM) (25 mM), catalase (bovine liver, 1500 units, Sigma Chemical Co., St. Louis, MO); tyramine (10 mM). In some experiments, $CuSO_4$ was used in place of NEM. The reaction was carried out at 37° for 1 hour and was terminated by the addition of 0.2 ml of 3 M trichloroacetic acid. Several blanks were investigated: a reaction omitting NEM or $CuSO_4$; a complete mixture using boiled enzyme; or a complete mixture containing fusaric acid (0.1 mM), a DBH inhibitor. The most suitable blank was found to be one employing fusaric acid. Additional samples were run in which a fixed amount of octopamine, the product of the reaction, was added to blank reaction mix-

[4] Brain tissue for both mice and rats does not include the olfactory lobes. Rat brain tissue does not contain the cerebellum, which was removed for enzyme determinations.

TABLE 1

Body weight, brain weight, hemoglobin and ceruloplasmin levels of dietary and genetic copper-deficient mice and their controls[1]

	+Cu	−Cu	$Mo^{+/y}$	$Mo^{br/y}$
Body weight, *g*	5.87 ± 0.45 (16)[a]	3.25 ± 0.42 (23)[b]	6.63 ± 0.66 (19)[c]	3.70 ± 0.52 (18)[d]
Brain weight, *mg*	315 ± 14.5 (6)[a]	234 ± 11.4 (7)[b]	332 ± 13.6 (13)[a]	273 ± 18.3 (12)[c]
Hemoglobin, *g/dl*	9.79 ± 0.92 (11)[a]	7.72 ± 0.77 (13)[b]	9.17 ± 0.70 (11)[a]	11.4 ± 1.02 (11)[c]
Ceruloplasmin,[2] $\mu mol \cdot min^{-1} \cdot liter^{-1}$	14.3 ± 1.3 (12)[a]	1.29 ± 1.15 (14)[b]	15.3 ± 2.0 (14)[a]	1.95 ± 0.63 (13)[b]

[1] Values are means ± SD for the number (in parentheses) of 11- to 12-day-old male C57BL mice. Within a row, numbers not sharing a common superscript are significantly different by Student's *t*-test ($P < 0.01$). Dietary copper deficiency was produced in offspring of normal dams fed a diet low in copper (−Cu). Some dams were given copper in the drinking water, and their offspring served as controls (+Cu). Genetic copper deficiency was studied in male offspring ($Mo^{+/y}$ and $Mo^{br/y}$) of dams heterozygous for the brindled allele at the mottled locus of the X chromosome ($Mo^{+/br}$) which were bred to normal males ($Mo^{+/y}$). These mice were fed a nonpurified stock diet and tap water. [2] Ceruloplasmin was estimated by measuring the ability of serum to oxidize *o*-dianisidine.

tures to correct for product recovery. After the assay, the supernatant fraction was applied to small glass columns packed with 0.8 ml of a slurry containing 0.33 g of Dowex 50W-X4 (200–400 mesh) per ml of 0.1 *N* HCl. The octopamine was eluted and oxidized with $NaIO_4$ according to Kato et al. (17). Excess $NaIO_4$ was removed by addition of 0.01 ml of $NaHSO_3$ (5% wt/vol). The *p*-hydroxybenzaldehyde was extracted into diethylether and back-extracted into NH_4OH before the absorbance was measured at 333 nm and 360 nm. Under these conditions the formation of octopamine was linear for at least 90 minutes, and the recovery of product was linear up to 16 nmoles.

Several noncuproenzymes were estimated in brain. Fumarase (EC 4.2.1.2) activity was measured spectrophotometrically by the method of Racker as previously described (5). The myelin-enriched protein 2′:3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (EC 3.1.4.37) was assayed as described previously (18) with slight modifications. The assay mixture contained 20 μg of purified alkaline phosphatase (*Escherichia coli*), and the reaction was terminated by addition of 0.1 ml of cold 15% trichloroacetic acid. Glutathione peroxidase (EC 1.11.1.9) (GSH-Px) activity was determined spectrophotometrically at 340 nm by a coupled enzyme procedure with glutathione reductase, as described elsewhere (19), except that tert-butylhydroperoxide (0.5 mM) (K & K Laboratories, Inc., Plainview, NY) was used as a substrate, the EDTA concentration was reduced to 0.1 mM, and the temperature was raised to 37°. Most biochemicals were obtained commercially (Sigma Chemical Co.).

Statistical analysis. Population means were compared using the *F*-variance ratio and Student's *t*-test by means of a computer program, Statistical Package for the Social Sciences (SPSS). Statistical significance was tested at $P = 0.01$.

RESULTS

Growth and development. Production of dietary copper deficiency in C57BL mice was evaluated and compared to genetic copper deficiency (brindled mice) in young male mice (table 1). The dietary treatment during perinatal development produced copper-deficient signs in the male offspring. In agreement with similar studies employing mice of another strain (C3H/HeJ) (14), the dietary deficient mice (−Cu) were anemic, whereas the brindled mice ($Mo^{br/y}$) were not, despite the fact that both groups of mice had greatly depressed ceruloplasmin activities, 9 and 13% of their respective controls (+Cu, $Mo^{+/y}$). In contrast, however, the −Cu mice of the C57BL strain exhibited a large growth deficit (45%) compared to copper-supplemented offspring (+Cu) (table 1), whereas body weights of −Cu mice of the C3H/HeJ strain were not different from +Cu body weights (14). Brindled mice of the C57BL strain had growth deficits similar to the −Cu mice (45%) (table 1) but less than the brindled mice of the C3H/HeJ strain (61%).

TABLE 2

Brain copper and iron levels of dietary and genetic copper-deficient mice and their controls[1]

Mouse strain	+Cu	−Cu	$Mo^{+/y}$	$Mo^{br/y}$
		Copper, μg/g		
C3H/HeJ	1.21 ± 0.04 (4)[a]	0.293 ± 0.100 (3)[b]	1.33 ± 0.11 (5)[a]	0.431 ± 0.076 (3)[b]
C57BL	1.23 ± 0.20 (4)[a]	0.272 ± 0.122 (4)[b]	1.24 ± 0.13 (8)[a]	0.382 ± 0.152 (6)[b]
		Iron, μg/g		
C3H/HeJ	6.38 ± 0.51 (4)[a]	6.36 ± 1.44 (3)[a]	9.18 ± 0.61 (5)[b]	13.6 ± 1.34 (4)[c]
C57BL	7.72 ± 0.28 (2)[a]	8.60 ± 0.43 (4)[a]	7.76 ± 1.67 (8)[a]	9.80 ± 4.39 (5)[a]

[1] Values are means ± SD for the number (in parentheses) of 11- to 12-day-old male mice. Within a row, numbers not sharing a common superscript are significantly different by Student's *t*-test ($P < 0.01$). Metal analyses were performed by flame atomic absorption spectroscopy following wet-digestion in nitric acid. Data are expressed on the basis of fresh tissue weight.

The strain differences in growth (body weight) were also reflected in changes in brain weight. The −Cu C57BL mice had significant decreases in whole brain weight (26%) compared to +Cu mice. The reduction was greater than that found in $Mo^{br/y}$ mice (18%), compared to their controls ($Mo^{+/y}$). In fact, the brain weights of $Mo^{br/y}$ mice exceeded those of −Cu mice (table 1). In C3H/HeJ mice, where −Cu body weights were normal, the situation was quite different. The average brain weight of the +Cu mice, 364 ± 14.5 mg [17] (mean ± SD [number of mice]), was not different from the −Cu mice (357 ± 19.4 [18]). However, the brain weight of the $Mo^{br/y}$ offspring was greatly reduced (240 ± 23.4 [9]) compared to the $Mo^{+/y}$ mice (345 ± 16.4 [14]) ($P < 0.01$).

The more severe copper-deficient state of C57BL resulted in a higher incidence of morbidity. Most −Cu C57BL offspring did not survive the suckling period, in contrast to −Cu C3H/HeJ mice (14). Brindled mice of both strains died at about 2 weeks of age when not used in the present studies. Copper supplementation to the drinking water of dams resulted in offspring (+Cu) which appeared normal in most respects (brain weight, hemoglobin, ceruloplasmin). However, the +Cu mice were significantly smaller (11%) than $Mo^{+/y}$ mice (table 1). No such difference in body weight was evident in C3H/HeJ control mice (+Cu, $Mo^{+/y}$) (14).

Copper and iron levels. Some offspring brains were wet-digested, and the content of total copper and iron was determined (table 2). The dietary copper deficiency in both strains of mice resulted in offspring (−Cu) with greatly diminished levels of brain copper (24 and 22%), compared to supplemented offspring. The levels of brain copper in $Mo^{br/y}$ mice of both strains was also low (32 and 31%), compared to normal littermates (table 2). The brain iron levels of dietary and genetic copper-deficient mice were not lower than control values. In fact, they tended to be elevated on occasion, but there was much variation between animals (table 2).

Dietary copper deficiency was produced in rats in two separate experiments and offspring from several litters were randomly sampled at 12, 20 and 34 days of age. In these studies, body weights of the −Cu offspring at 12 and 34 days of age were not greatly different from +Cu body weights, 92% and 91% of +Cu offspring, respectively, whereas at 20 days of age the −Cu rats weighed less, 63% of +Cu (14). Brain was analyzed for total copper and iron during this perinatal period in −Cu offspring, as well as in offspring from supplemented dams (+Cu) (fig. 1). Dietary copper deficiency greatly reduced brain copper levels at all ages and significantly reduced brain iron as well ($P < 0.01$). However, the quantitative reductions in rat brain iron (16–26%) were much less than the corresponding reductions in copper levels (70–74%).

Catecholamine analysis. Several experiments were conducted in which both dietary

and genetic copper deficiency was produced in mice. Male mice from several litters were sampled and brains were analyzed for norepinephrine and dopamine content by reverse-phase ion-pair liquid chromatography with electrochemical detection. This highly selective and sensitive technique offers many analytical advantages. Brindled mice and −Cu mice of both mouse strains demonstrate greatly reduced brain norepinephrine concentrations (table 3). The deficit appeared most severe in C3H/HeJ $Mo^{br/y}$ mice (8% of control) and C57BL −Cu mice (13% of control) which corresponded well with the deficit in brain weight in these two groups. Norepinephrine values in control mice of both strains were similar (table 3). In contrast, the brain concentrations of dopamine were normal in $Mo^{br/y}$ mice and −Cu mice. In fact, in one strain, C3H/HeJ, dopamine was elevated in −Cu mice (table 3). Dopamine values in control mice of both strains were similar.

Catecholamines were also analyzed in rat brain to compare with the mouse model (fig. 2). In agreement with the −Cu mice data, the brain norepinephrine concentration was lower in −Cu rats (ages 12 and 20 days) than in +Cu rats. However, at no age was dopamine significantly altered in concentration (fig. 2) in agreement with the mouse data (table 3).

Enzyme activities. A functional deficit in mouse brain copper metabolism, anticipated because of the low brain copper levels (table 2) and suggested by the depressed norepinephrine concentrations (table 3), was expressed in both −Cu and $Mo^{br/y}$ brain cytochrome oxidase activities (table 4). The depressed cytochrome oxidase activities in −Cu mice (18.5 and 18.8% of +Cu values) were equivalent to those in $Mo^{br/y}$ mice (21.4 and 25.5% of $Mo^{+/y}$ values) (table 4). Control mice of both strains had similar activities. Cytochrome oxidase activities were also reduced in −Cu rat brain, but to a lesser degree; the −Cu values were 27, 29 and 58% of +Cu activities at 12, 20 and 34 days of age, respectively.

The activity of a second cuproenzyme, DBH, was measured in C57BL mouse brain to extend the catecholamine analyses. The activity of DBH was elevated in both −Cu and $Mo^{br/y}$ brains by 25 and 51%, respectively (table 4). This increase was verified in five separate experiments (Prohaska, J. and Cox, D., unpublished data). The DBH activity was determined in the presence of *N*-ethylmaleimide (NEM), which was necessary to stimulate activity (fig. 3). The stimulation was not due to extraneous copper addition, since Cu analysis of the reaction mixture (without brain tissue) yielded a value of 0.8 μM, a Cu level far below that required for stimulation (fig. 3). The elevation in DBH activity was observed in −Cu and $Mo^{br/y}$ samples when either copper or NEM was used (fig. 3). However, at low levels of NEM or copper, the

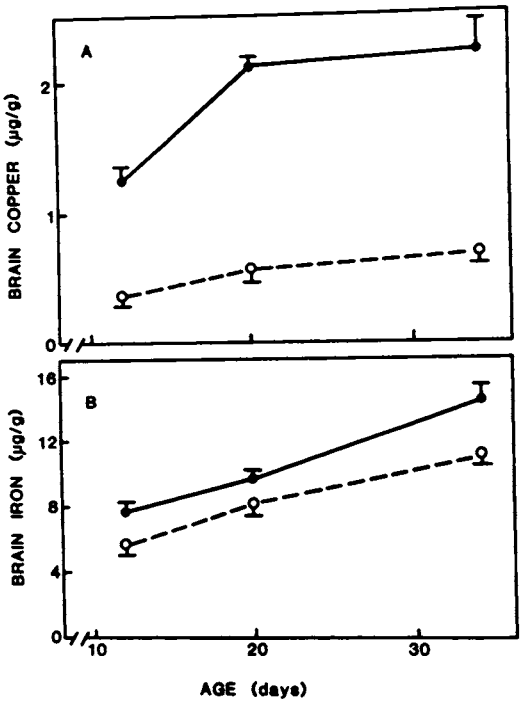

Fig. 1 Rat brain copper (1A) and iron (1B) concentrations during perinatal copper deficiency. Copper deficiency was produced by feeding a purified diet low in copper to dams during gestation and lactation. Some dams were supplemented with copper in the drinking water. At ages 12, 20 and 34 days, four to seven control (●) and copper-deficient (○) offspring were randomly selected from at least four litters in two separate experiments. Brains were removed and wet-digested in HNO_3, and the copper and iron contents were determined by flame atomic absorption spectroscopy. Each point represents the mean ±SD. At all ages for both metals, the means were significantly different by Student's *t*-test ($P < 0.01$).

TABLE 3

Brain norepinephrine and dopamine levels of dietary and genetic copper-deficient mice and their controls[1]

Mouse strain	+Cu	−Cu	$Mo^{+/y}$	$Mo^{br/y}$
		Norepinephrine, ng/g		
C3H/HeJ	270 ± 82.8 (4)[a]	56.2 ± 31.6 (7)[b]	183 ± 33.7 (8)[c]	14.5 ± 15.5 (4)[b]
C57BL	256 ± 33.8 (5)[a]	33.0 ± 20.0 (5)[b]	218 ± 40.7 (8)[a]	37.3 ± 27.8 (9)[b]
		Dopamine, ng/g		
C3H/HeJ	268 ± 40.9 (4)[a]	341 ± 48.8 (7)[b]	310 ± 43.7 (8)[a]	261 ± 38.2 (4)[a]
C57BL	297 ± 112 (5)[a]	388 ± 125 (5)[a]	292 ± 50.3 (8)[a]	307 ± 74.0 (9)[a]

[1] Values are means ± SD for the number (in parentheses) of 11- to 12-day-old male mice. Catecholamine analysis was performed by electrochemical detection of the amines separated by reverse-phase liquid chromatography with ion-pairing. Data are expressed on the basis of fresh tissue weight. Within a row, numbers not sharing a common superscript are significantly different by Student's *t*-test ($P < 0.01$).

control (+Cu or $Mo^{+/y}$) DBH activities were equivalent to or exceeded the deficient (−Cu or $Mo^{br/y}$) brain activities. Thus, more copper or NEM was required in the copper-deficient DBH assays to produce maximal activities (fig. 3).

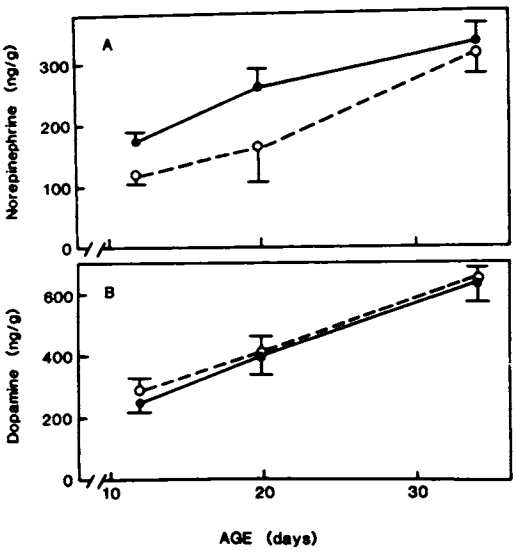

Fig. 2 Rat brain norepinephrine (2A) and dopamine (2B) during perinatal copper deficiency. Copper deficiency was produced by feeding a purified diet low in copper to dams during gestation and lactation. Some dams were supplemented with copper in the drinking water. At ages 12, 20, and 34 days, six to eleven control (●) and copper-deficient (○) offspring were randomly selected from at least four litters in two separate experiments. Brains were removed and the cerebellum was discarded. The remainder was analyzed for catecholamines by HPLC with electrochemical detection. Each point represents the mean ±SD. When analyzed by Student's *t*-test, brain norepinephrine levels were different at ages 12 and 20 days ($P < 0.01$). Other means were not significantly different.

Since DBH activity in vitro did not explain the low brain norepinephrine levels in −Cu and $Mo^{br/y}$ mice, further enzymatic analysis was initiated. Recently (21), it was reported that dietary copper deficiency resulted in a decrease in the activity of the selenoenzyme GSH-Px. In brain, GSH-Px may be necessary to remove H_2O_2 to augment the DBH reaction, just as catalase does in vitro. Therefore, brain GSH-Px activity was determined in our experiments (table 4). Neither dietary or genetic copper deficiency had any significant effect on brain GSH-Px activity.

The depressed growth of the brain was particularly evident in C57BL −Cu mice and C3H/HeJ $Mo^{br/y}$ mice. The brains of these groups of mice demonstrated a significant lag in myelination as evidenced by reduction in the activity of CNP, a myelin-associated protein (table 4). However, other copper-deficient mice (−Cu C3H/HeJ mice and the $Mo^{br/y}$ C57BL mice) had CNP activities equivalent to their respective controls, indicating the selective nature of the CNP deficit. The CNP reduction that was observed did not simply reflect a decrease in all brain enzymes, since fumarase activity was normal in all −Cu and $Mo^{br/y}$ mice when compared to their respective controls (Prohaska, J. and Smith, T., unpublished).

DISCUSSION

The present studies, comparing dietary and genetic copper deficiency, were con-

TABLE 4

Brain enzyme activities of dietary and genetic copper-deficient mice and their controls[1]

Mouse strain	+Cu	−Cu	$Mo^{+/y}$	$Mo^{br/y}$
	Cytochrome oxidase, $\mu mol \cdot min^{-1} \cdot mg^{-1}$			
C3H/HeJ	0.466 ± 0.018 (4)[a]	0.086 ± 0.005 (3)[b]	0.448 ± 0.081 (3)[a]	0.096 ± 0.033 (3)[b]
C57BL	0.400 ± 0.036 (4)[a]	0.075 ± 0.011 (4)[b]	0.392 ± 0.066 (4)[a]	0.100 ± 0.018 (4)[b]
	Dopamine-β-hydroxylase, $nmoles \cdot hr^{-1} \cdot mg^{-1}$			
C57BL	4.22 ± 0.40 (4)[a]	5.26 ± 0.60 (4)[b]	4.12 ± 0.64 (5)[a]	6.24 ± 1.08 (6)[b]
	Glutathione peroxidase, $\mu mol \cdot min^{-1} \cdot mg^{-1}$			
C3H/HeJ	—	0.0418 ± 0.0028 (4)[a]	0.0462 ± 0.0073 (6)[a]	0.0551 ± 0.0147 (3)[a]
C57BL	0.0690 ± 0.0044 (3)[a]	0.0706 ± 0.0039 (4)[a]	0.0627 ± 0.0022 (3)[a]	0.0617 ± 0.0094 (3)[a]
	2',3'-Cyclic nucleotide 3'-phosphodiesterase, $\mu mol \cdot min^{-1} \cdot mg^{-1}$			
C3H/HeJ	0.774 ± 0.136 (8)[a]	0.609 ± 0.264 (6)[a]	0.739 ± 0.043 (3)[a]	0.300 ± 0.059 (3)[b]
C57BL	1.01 ± 0.021 (4)[a]	0.579 ± 0.093 (4)[b]	0.782 ± 0.0852 (4)[c]	0.764 ± 0.037 (4)[c]

[1] Values are means ± SD for the number (in parentheses) of 11- to 12-day-old male mice. Most activities are expressed on the basis of micromoles per minute per milligram of total brain protein. Dopamine-β-hydroxylase activity was measured on a portion of the brain that excluded the cerebellum and cerebrum. Numbers within a row not sharing a common superscript are significantly different by Student's *t*-test ($P < 0.01$).

ducted with two species of animals, rats and mice, and with two strains of mice C57BL and C3H/HeJ. There was a major contrast in the susceptibility to dietary copper deficiency in mice between the strains and a modest contrast in the genetic offspring, as evidence by growth and morbidity data. Biochemical analyses of several copper-dependent factors (ceruloplasmin, hemoglobin and tissue copper) were similar in both strains of mice and reflected the respective copper status. Despite some differences in mouse strains, several conclusions are evident concerning the effect of perinatal copper deficiency on brain in mice and rats. First, both dietary and genetic copper deficiency result in impaired brain growth and greatly diminished levels of copper in brain. Low brain copper levels have previously been reported in studies with rats (4, 5) and brindled mice (11). Second, the low brain copper levels are associated with a functional deficit in the copper-dependent enzyme cytochrome oxidase. Copper-deficient rats (4–6) and brindled mice (12) were known to exhibit decreases in brain cytochrome oxidase activities from previous work. Third, copper deficiency results in significant decreases in brain norepinephrine levels, whereas dopamine concentrations remain largely unaltered. This confirms previous work with brindled mice (9–11) but is in partial contrast to earlier work on dietary copper deficiency in rats. The results of this study suggest that the brain of the brindled mouse is similar to that of the −Cu mouse, that is, a brain characterized by low copper levels rather than by abnormal metabolism of the copper that is present.

Dietary copper deficiency in mice results in a significant lowering of brain norepinephrine concentrations, which confirms earlier work with rats (4–7) and lambs (8). However, the current studies do not agree with previous work (6—8) in which dopamine levels were reported to be lowered by copper deficiency. Species' differences do not account for the disparity, since we have demonstrated normal dopamine levels in rats as well as in mice. Age of the animals is not a factor, because other studies in copper-deficient C58 mice (22) that were the same age as rats with low dopamine levels (6, 7) demonstrated normal dopamine but low norepinephrine levels (16). The basal diet in the present studies has more sucrose and less lactose than that used by others (6), which may

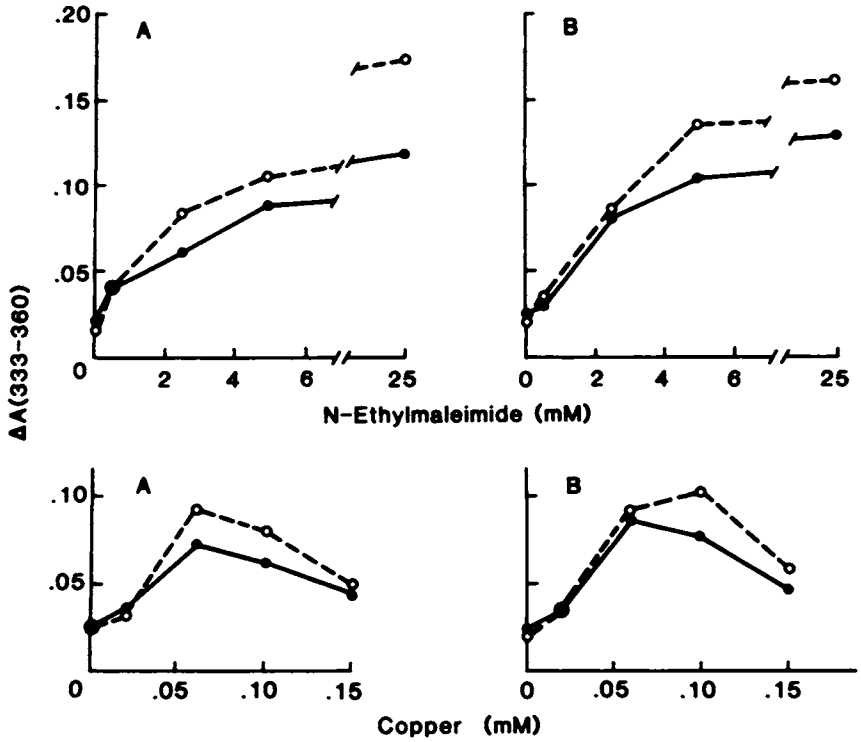

Fig. 3 Effect of varying $CuSO_4$ and *N*-ethylmaleimide (NEM) on the activity of mouse brain dopamine-β-hydroxylase (DBH). Pooled brain extracts from four 11- to 12-day-old male C57BL mice that were born to heterozygous brindled females, 3A, (○, $Mo^{br/y}$; ●, $Mo^{+/y}$) or normal females, 3B, fed a purified diet low in copper (○, −Cu; ●, +Cu) were assayed for DBH while varying the concentrations of the activators, $CuSO_4$ or NEM. The assay for DBH was based on measurement of *p*-hydroxybenzaldehyde by the difference in absorbance at 333 nm and 360 nm (17). The plotted values with no $CuSO_4$ or NEM added were equivalent to those in which boiled enzyme or fusaric acid, a DBH inhibitor, was added.

be a factor in the development of copper deficiency. The rats in the current study did have low brain copper levels and cytochrome oxidase activities, yet they may not have been as deficient as the rats studied previously by others (6, 7). If a more severe copper deficiency were employed, perhaps dopamine levels would be lower. The depression in rat brain dopamine levels is not reversed by copper repletion (7), which suggests that the loss in dopamine may be due to different mechanisms than norepinephrine, since the latter catecholamine does respond to copper repletion (5, 6).

The most likely explanation for low brain norepinephrine concentrations in copper-deficient animals is decreased synthesis resulting from diminished activity of DBH, a cuproenzyme. However, this is difficult to prove. When DBH was assayed, in vitro, in the presence of either copper or NEM, the activity in brain samples of both brindled mice and −Cu mice was elevated. Hunt first reported this observation in brindled mice (11) when copper was added, and feels that activation of apoenzyme may explain the apparent DBH overshoot (23). Recently, Hesketh has reported that DBH activity in adrenal glands of copper-deficient rats was elevated compared to controls, and NEM was used to inactivate the endogenous inhibitor (24). Hesketh argues that deprivation of norepinephrine cannot be accounted for by reduced DBH activity but that it may be due to a defect in storage or an increase in catabolism or reduction in synthesis. Storage of norepinephrine during a copper-deficient state has not yet been examined, but catabolism and synthesis have.

Monoamine oxidase, a catecholamine cat-

abolic enzyme, is not altered by dietary copper deficiency in rat brain (5), lamb brain (8), or in brindled mouse brain (10). Also, catechol-*O*-methyl transferase activity is normal in brindled mouse brain (23), suggesting no increase in catabolism. The levels of brain tyrosine are normal in −Cu rats (7) and elevated in brindled mice (9, 10), ruling out precursor levels as an explanation for low norepinephrine. Furthermore, tyrosine hydroxylase, the rate-limiting enzyme in norepinephrine synthesis, was reported to be elevated in brindled mouse brain (10).

Studies on the conversion of [^{3}H]tyrosine into [^{3}H]dopamine and [^{3}H]norepinephrine in brindled mice (10) are consistent with a block in DBH, since dopamine was higher and norepinephrine lower in mutant mouse tissue. Similar studies in vivo with −Cu rats in cardiac tissue also suggest a functional deficiency in DBH activity (25). Rohmer et al. (26) have measured DBH activity in a postmortem sample of the frontal cortex from a subject who died of Menkes' disease. In contrast to the work in rodents, they found a decrease in DBH activity which persisted even when exogenous copper was added. Thus, depressed DBH activity may be responsible for the decrease in norepinephrine levels.

The disparity between in vitro and in vivo DBH activity is puzzling. In mouse brain, no DBH activity is detected in vitro unless an activator, such as Cu^{2+} or NEM, is added, a situation unlikely to occur in vivo. Estimates of in vitro DBH activity may be useful in predicting induction of enzyme (both active DBH and apoenzyme) but do not reflect endogenous norepinephrine synthesis. Pharmacological depletion of norepinephrine leads to an increase in DBH activity (27), and thus a similar feedback situation might occur in copper deficiency and explain the DBH overshoot. There is certainly enough residual copper, even in the −Cu and $Mo^{br/y}$ mouse brain, to account for normal DBH activity. (If one assumes that a unit of activity of purified DBH and that measured in crude extracts represents an equivalent amount of enzyme, then mouse brain DBH accounts for only 0.01% of the total copper pool). The activity of DBH is elevated, in vitro, in −Cu and $Mo^{br/y}$ mouse brain preparations. However, the in vivo isotope studies (10, 25) indicate that functional DBH activity is low in −Cu tissues. A possible explanation for these observations is that copper deficiency, both dietary and genetic, leads to a decrease in DBH activity in vivo, which results in a reduction in norepinephrine synthesis. The depletion of norepinephrine induces more apo-DBH synthesis, which becomes activated in vitro by trace amounts of copper used in preparing the samples and assaying the DBH activity. Alternatively, the −Cu and $Mo^{br/y}$ tissues might contain higher levels of an endogenous inhibitor of DBH, which would reduce activity in vivo. Since higher levels of Cu^{2+} or NEM were necessary to express full DBH activity in vitro, there is some evidence for this.

Other factors may account for low brain norepinephrine in copper-deficient brain. Copper status may influence brain ascorbate levels. Chronic ascorbic acid deficiency in guinea pigs is known to reduce brain norepinephrine levels (28). In the present study, copper deficiency in rats resulted in significant reductions in brain iron, which confirms earlier work (5). Iron is a likely cofactor of tyrosine hydroxylase, the first enzyme in norepinephrine synthesis. The activity of tyrosine hydroxylase was measured in −Cu rat brain and found to be reduced (6). This may result from neuronal loss rather than from iron depletion. Several facts may be used to argue against a specific role for iron in brain catecholamine metabolism in −Cu animals: *1*) brain iron levels are normal in −Cu mice and $Mo^{br/y}$ mice, 2) tyrosine hydroxylase activity is elevated in $Mo^{br/y}$ mice (10); and 3) no changes in brain dopamine or norepinephrine were found in iron-deficient rat brain (29). Adequate copper may be necessary for normal selenium metabolism, as reflected in GSH-Px activities (21). In $Mo^{br/y}$ and −Cu mouse brain, the activity of GSH-Px is equivalent to control values. Thus, altered GSH-Px and H_2O_2 levels would seem unlikely to be involved in norepinephrine depletion.

It seems likely that the decrease in brain norepinephrine levels is specific to copper depletion and not a general effect of brain atrophy. Perinatal malnutrition induced by protein deficiency is actually accompanied

by increases in brain norepinephrine levels (30). This nutritional treatment also reduces the number of α- and β-adrenergic receptor sites (31). Dietary copper deficiency, in contrast, does not alter β-adrenergic receptors but does decrease both dopamine and muscarinic receptors in certain brain regions (32). Clearly, the effect of copper deficiency on catecholamine metabolism is selective.

The decrease in norepinephrine accompanying copper deficiency may be partly responsible for the altered morphology (5, 6, 13) and function of the copper-deficient brain. In vitro studies have shown that norepinephrine can induce synthesis of CNP, an oligiodendroglial plasma membrane protein, by binding to a β-adrenergic receptor (33). Thus, it is possible that the hypomyelination observed in copper-deficient brain may be the result of low norepinephrine levels. Further work is necessary to determine the exact cause for norepinephrine depletion in copper deficiency and the consequences of this depletion.

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