Comparisons of copper deficiency states in the murine mutants blotchy and brindled

Changes in copper-dependent enzyme activity in 13-day-old mice

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The activity of two copper-dependent enzymes, cytochrome *c* oxidase and copper,zinc-superoxide dismutase, was determined in six tissues of age-matched (13-day-old) copper-deficient mutant and normal mice. In the two mutants 'brindled' and 'blotchy', brain, heart and skeletal muscle had significant enzyme deficiencies. Cytochrome *c* oxidase was more severely affected than was superoxide dismutase. In these three tissues the degree of deficiency could be correlated with decreased copper concentration; however, enzyme activity was normal in liver, kidney and lung, despite abnormal copper concentrations in these tissues. In nutritionally copper-deficient mice, all six tissues showed decreased enzyme activity, which was most marked in brain, heart and skeletal muscle, the tissues which showed enzyme deficiencies in the mutants. Analysis *in vitro* of cytochrome *c* oxidase (temperature coefficient = 2) at a single temperature was found to underestimate the deficiency of this enzyme in hypothermic copper-deficient animals. Cytochrome *c* oxidase deficiency may therefore be sufficiently severe *in vivo* to account for the clinical manifestations of copper deficiency. An injection of copper (50 µg of Cu⁺) at 7 days increased cytochrome *c* oxidase activity by 13 days in all deficient tissues of brindled mice, and in brain and heart from blotchy mice. However, skeletal-muscle cytochrome *c* oxidase in blotchy mutants did not respond to copper injection. Cytochrome *c* oxidase activity increased to normal in all tissues of nutritionally copper-deficient mice after copper injection, except in the liver. Hepatic enzyme activity remained severely deficient despite a liver copper concentration three times that found in copper-replete controls. Superoxide dismutase activity did not increase with treatment in either mutant, but its activity was higher than control levels in nutritionally deficient mice after injection. This difference is probably due to sequestration of copper in mutant tissue such as kidney, but a defect in the copper transport pathway to superoxide dismutase cannot be excluded.

INTRODUCTION

The pleiotropic effects of both dietary and genetic copper deficiency may be ascribed to decreased activity of a number of copper-dependent enzymes.

The X-chromosome-linked murine mutations 'brindled' and 'blotchy' have defects in copper homeostasis (Hunt, 1974) resulting in an abnormal distribution of the trace element to tissues within the body (Hunt, 1974, 1976, 1977; Mann *et al.* 1979*a,b*). Copper accumulates in some tissues, for example kidney, and is deficient in others, including brain.

The abnormalities in tissue copper distribution are qualitatively similar in the two mutants, although the magnitude of the defect is greater in the brindled mouse. Surprisingly, the pathology of the animals is very different.

The brindled mutant dies before weaning with signs of profound neurological disturbance that have been attributed to a deficiency of copper-dependent enzymes in brain, including cytochrome *c* oxidase (EC 1.9.3.1), SOD (EC 1.15.1.1) (Danks, 1977) and dopamine β-hydroxylase (EC 1.14.17.1) (Hunt, 1977; Danks, 1977). The blotchy mutant dies at a mean age of 200 days with connective-tissue abnormalities apparently resulting

from a lysine oxidase ('lysyl') (EC 1.4.3.13) deficiency (Rowe *et al.*, 1977; Royce *et al.*, 1982).

There have been a number of studies of copper-dependent enzyme activity in tissues of these mice (Hunt, 1974, 1977; Rowe *et al.*, 1974, 1977; Wenk & Suzuki, 1983; Prohaska, 1983), but these have either involved the study of one mutant only, or, where both mutants were studied, the mice have been at different stages of development. Since there are significant changes in enzyme activity during development, age-matched studies are likely to result in more meaningful comparisons between the mutants.

Royce *et al.* (1982) investigated lysine oxidase activity in age-matched brindled and blotchy newborns and reported a greater decrease in enzyme activity in the more viable blotchy mutant. Treatment with a subcutaneous copper injection increased enzyme activity to normal levels in the skin of brindled mutants, but had little effect on this enzyme in blotchy mice. This data is consistent with the differential pathology of the two mutants and with the more favourable response to treatment reported for the brindled mutant (Mann *et al.*, 1979*a*, 1981). Phillips *et al.* (1981; M. Phillips, J. Camakaris, J. Mann & D. M. Danks, unpublished work) found significant increases in the activity of both cytochrome *c* oxidase and

Abbreviation used: SOD, superoxide dismutase.

SOD during postnatal development in some tissues of normal mice. These increases were absent or reduced in mutant mice. It seemed likely that a failure to meet normal developmental enzyme requirements might explain the early death of the brindled mutant. In addition, the differences in response to copper treatment seen in the mutants, and the variations in pathology, may be reflected in tissue-specific differences in copper-dependent enzyme activity and copper concentration. Thus functional copper pools could be distinguished from total copper.

In the present paper the activities of cytochrome *c* oxidase and SOD are compared in six tissues of 13-day-old brindled and blotchy mutants. Copper-replete and copper-deficient (Cu⁻) genetically normal mice were used as controls. The effect of subcutaneous copper injection at 7 days on 13-day-old animals was also determined.

The results reported show a marked similarity in extrahepatic cytochrome *c* oxidase activity and response to treatment in brindled mutants and Cu⁻ mice. In contrast, SOD deficiency and response to treatment was very different in genetic copper deficiency and nutritional copper deficiency. Injected copper may not be available to skeletal-muscle cytochrome *c* oxidase in the blotchy mutant.

EXPERIMENTAL

Animals

Hemizygous mutant male mice were obtained by crossing heterozygous females carrying either the brindled (*br*/+) or the blotchy (*blo*/+) mutation with normal males (+/y).

Genetically normal mice obtained from these crosses were mated and fed a low-copper diet from the day of mating. The offspring produced were copper-deficient and were used as controls in comparisons with the genetically copper-deficient mutants. Copper-replete normal mice were also assayed.

All mice were assayed when 13 days old. Only healthy mice were assayed.

Diet

All mice not involved in nutritional experiments were fed commercial rat and mouse cubes (Mecon), containing 17.8 µg of copper/g, 95.6 µg of zinc/g and 341 µg of iron/g by analysis, and acidified tap water *ad lib*.

The low-copper diet was prepared according to the American Institute of Nutrition Standards for Nutritional Studies Report (Report of the American Institute of Nutrition, 1977) with CuCO₃ omitted from the mineral mix (0.37 µg of copper/g, 32.0 µg of zinc/g and 33.0 µg of iron/g by analysis). Distilled water was given *ad lib*. Control mice for nutritional experiments were fed the same diet, but were supplemented with 20 p.p.m. of Cu²⁺ (as CuSO₄) in the drinking water.

Copper treatment procedures

Mice were treated with a subcutaneous injection of copper at 7 days of age. Treatment doses of 50 µg copper/10 µl [as Cu⁺ complexed to sebatic acid in 10% (v/v) alkyl polyether and 90% (v/v) propane-1,2-diol] were administered by using a 10 µl calibrated glass syringe with a 2.5 cm needle.

The Cu⁺ sebatic acid solution was used to allow comparisons with previous studies by Camakaris *et al.* (1979) and Mann *et al.* (1979a,b, 1981).

Enzyme assays

Tissue preparation. Mice were anaesthetized with an intraperitoneal injection of pentobarbitone (Nembutal) according to body weight, and perfused with ice-cold sterile saline (0.9% NaCl). Six tissues (liver, brain, heart, kidney, lung and skeletal-muscle sample) were removed and weighed.

The weighed tissues were homogenized (1:5, w/v) in 0.02 M-Tris/HCl/10 µM-EDTA/0.2% Triton X-100, pH 7.5, by using Dounce glass/glass homogenizers. Skeletal muscle was resistant to homogenization and, to ensure more reproducible extraction of the mitochondrial enzyme, the muscle was pre-treated with a relaxant buffer (Zak *et al.*, 1970).

Homogenates were kept on ice for cytochrome *c* oxidase assays. A sample was removed for copper analysis and the remainder stored at -20 °C for SOD studies.

Cytochrome *c* oxidase. Cytochrome *c* oxidase activity was assayed by the method of Smith (1955) as modified by Errede *et al.* (1978). The oxidation of reduced cytochrome *c* (50 µM; Sigma grade 111) by mouse tissue extracts was monitored spectrophotometrically at 550 nm and at 25 °C. The assay temperature was chosen because minor fluctuations in temperature around 25 °C had little effect on enzyme activity (Fig. 1 below). The extracts were diluted to give a linear initial reaction rate. The spectrophotometer used was a Pye-Unicam SP.1800 fitted with a Unicam AR linear recorder and a thermostatically controlled Haake F. J. water heater and pump. All assays were performed in duplicate and the mean result for the two assays taken as the result. Variation between duplicates was less than 5%.

All assays were completed within 12 h of tissue dissection. Frozen tissues and homogenates were not used.

The presence of 0.2% Triton X-100 in the homogenization buffer increased cytochrome *c* oxidase activity in the tissues assayed, but did not change the ratio of mutant to normal activity.

SOD. SOD was assayed in tissue homogenates at 25 °C and pH 10 by using the xanthine/xanthine oxidase method of McCord & Fridovich (1969) and 10 µM-acetylated cytochrome *c* (Azzi *et al.* 1975).

Contaminating manganese-SOD was removed by chloroform/ethanol extraction (Weisiger & Fridovich, 1973), and removal or inactivation was confirmed by electrophoresis of some tissue extracts on native 7.2%-(w/v)-acrylamide gels (Davis, 1964), followed by nitroretazolium staining [Beauchamp & Fridovich (1971) as modified by Salin & McCord (1974)].

All SOD values were calculated relative to a standard curve prepared by using commercially obtained bovine erythrocyte SOD (Sigma S 8254). By using this assay bovine SOD had an activity of 33300 units/mg of protein. (One SOD unit is defined as the concentration that will inhibit by 50% the rate of reduction of cytochrome *c*, i.e. from 0.02 to 0.01 absorbance unit under the assay conditions specified). Linearity of the assay was estimated for extracts of each tissue studied.

Table 1. Copper concentration in tissues of 13-day-old treated and untreated mice

Results are means $\pm$ S.E.M. (*n*) were analysed by a two-tailed Student's *t* test: ***P* < 0.01; ****P* < 0.001. Abbreviation used: (T), treated animals.

Genotype	Copper (μ g/g wet wt. of tissue)					
	Tissue . . . Liver	Brain	Heart	Kidney	Lung	Muscle
+ /y	33.8 $\pm$ 3.1(7)***	2.5 $\pm$ 0.3(6)***	3.16 $\pm$ 1.1(5)	3.7 $\pm$ 1.1(5)***	1.9 $\pm$ 0.5(5)	1.0 $\pm$ 0.4(5)*
br/y	6.4 $\pm$ 2.3(8)	0.7 $\pm$ 0.1(7)	1.62 $\pm$ 1.0(5)	13.1 $\pm$ 2.2(7)	1.6 $\pm$ 0.2(6)	0.7 $\pm$ 0.1(4)
+ /y(T)	115(2)		4.2(2)		2.5(2)	
br/y(T)	13.9 $\pm$ 3.4(3)	0.7 $\pm$ 0.1(3)	6.1 $\pm$ 2.2(3)	41.8 $\pm$ 0.2(3)	2.1 $\pm$ 0.4(3)	1.6(2)
+ /y	40.2 $\pm$ 3.6(5)***	1.8 $\pm$ 0.5(5)**	4.3 $\pm$ 1.2(5)*	2.5 $\pm$ 0.15(5)**	2.3 $\pm$ 0.7(5)	1.0 $\pm$ 0.3(4)
blo/y	11.0 $\pm$ 3.1(5)	0.7 $\pm$ 0.2(5)	1.43 $\pm$ 1.1(5)	7.0 $\pm$ 2.4(5)	1.6 $\pm$ 0.2(4)	0.8 $\pm$ 0.3(4)
+ /y(T)	85.4(2)	1.8(2)	3.5(2)	2.5(2)	1.6(2)	1.6(2)
blo/y(T)	14.6 $\pm$ 3.4(3)	1.3(2)	4.0–9.8(3)†	16.5 $\pm$ 2.9(3)	1.3(2)	(2)
Cu+	22.4 $\pm$ 1.8(6)***	1.8 $\pm$ 0.2(6)	6.0 $\pm$ 0.9(5)	2.5 $\pm$ 0.2(6)**	2.2 $\pm$ 0.4(6)	0.9 $\pm$ 0.2(6)
Cu–	8.6 $\pm$ 2.6(10)	1.3 $\pm$ 0.3(9)	4.8 $\pm$ 0.2(9)	1.5 $\pm$ 0.2(8)	1.6 $\pm$ 0.3(9)	0.7 $\pm$ 0.1(8)
Cu–(T)	81.2 $\pm$ 2.7(5)	1.5–5.9(5)†	4.5–17.5(5)†			

* *P* < 0.05 when littermates were analysed by using a paired *t* test.

† Owing to the wide variability in copper concentration in these tissues, the range of values is given rather than the mean $\pm$ S.E.M.

The reliability of the assay was tested by comparing SOD activity in diploid cultured fibroblasts 21, a monosomy 21 cell line (GM0230; NIGMS Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A.) and trisomy 18 cultures. The gene dosage was consistent with the activity obtained.

The data was analysed by Student's *t* test and Mann–Whitney non-parametric statistical tests.

RESULTS

Copper-deficient animals of all genotypes had reduced pigmentation and curled vibrissae. The pigmentation in Cu– mice was similar to that found in blotchy mutants. The mean age of death of brindled mutants in the colony was 17 days. Untreated Cu– mice died before 22 days of age, had pronounced neurological abnormalities and could be rescued by a single copper injection (50 μ g) at 7 days but not at 10 days.

The body weight of both mutants and of nutritionally deficient controls, Cu– mice, was significantly reduced (*P* < 0.05), but body length and tail length were normal when compared with the respective copper-replete controls. Injection with copper at 7 days did not significantly increase body weight at 13 days.

Tissue copper concentrations of mutant mice were abnormal (Table 1), as previously described (Hunt, 1974; Camakaris *et al.*, 1979; Mann *et al.*, 1979a,b, 1981). The data are included here to allow changes in enzyme activity to be related to alterations in copper levels in the population studied. A comparison with tissue copper concentrations in nutritionally copper-deficient (Cu–) age-matched controls indicates that the abnormal distribution in mutants is due to the mutations and not to copper deprivation (Table 1).

Cytochrome *c* oxidase activity.

Copper-replete normal mice. Cytochrome *c* oxidase activity was identical in copper-replete normal mice of both strains carrying the mutations and was largely unchanged in copper-supplemented mice fed the purified

copper-deficient diet (Cu+ mice) (Table 2). Liver cytochrome *c* oxidase was increased in mice fed the purified diet (*P* < 0.01), possibly because of a slightly more rapid development [cytochrome *c* oxidase activity increases during development (M. Phillips, unpublished work) and Cu+ mice were weaned at 17 days], maternal effects (mother normal rather than heterozygous for the blotchy or brindled mutation), better diet, or a combination of all three factors. Variability between individuals was greater in mice derived from females heterozygous for a mutation affecting copper homeostasis, presumably because of maternal effects, but the data, covering mice born to 18 mothers, suggests tight regulation of cytochrome *c* oxidase activity in the tissues studied.

Copper-deficient mice. 1. The mutants. As was previously reported (Phillips *et al.*, 1981), cytochrome *c* oxidase activity was decreased in the brain (*P* < 0.001), heart (*P* < 0.001) and skeletal muscle (*P* < 0.001) of brindled mice (Table 2). In contrast, lung, kidney and liver enzyme activity was within the normal range. The brain, kidney and liver data are consistent with previous reports (Hunt, 1977; Prohaska & Smith, 1982; Prohaska, 1983).

A similar pattern of tissue cytochrome *c* oxidase activity was found in the blotchy mutant. Brain (*P* < 0.001), heart (*P* = 0.024) and skeletal muscle (*P* < 0.05; paired *t* test) were significantly deficient, but the decrease in activity was less than that found in the brindled mouse.

In both mutants, tissues with reduced cytochrome *c* oxidase activity showed similar decreases in copper concentration (Table 2). When copper concentration was plotted against cytochrome *c* oxidase activity, a significant linear correlation was found for brain (*P* < 0.001, *n* = 17), heart (*P* < 0.05, *n* = 14) and skeletal muscle (*P* < 0.05, *n* = 13) for untreated normal and mutant mice. There was no correlation between enzyme activity and copper levels in those tissues with normal enzyme activity (Table 3).

Table 2. Cytochrome *c* oxidase activity in 13-day-old copper-deficient mice

Results are means $\pm$ S.E.M. (*n*) and were analysed by a two-tailed Student's *t* test. Abbreviation used: (T), treated animals. (Where the variances between samples were not equal, a separate variance estimate was used in determining the significance of data.)

Genotype	Cytochrome <i>c</i> oxidase activity (μ mol/min per g wet wt.)					
	Tissue . . . Heart	Liver	Kidney	Brain	Muscle	Lung
br+ /y	109.2 $\pm$ 9.4(6)***†	71.4 $\pm$ 8.7(8)	56.4 $\pm$ 10.7(5)	38.8 $\pm$ 3.1(7)***†	27.9 $\pm$ 2.1(7)***†	21.7 $\pm$ 1.9(7)
br/y	47.4 $\pm$ 3.0(5)***†	78.4 $\pm$ 7.7(8)	56.3 $\pm$ 3.3(5)	10.7 $\pm$ 0.4(7)***†	17.2 $\pm$ 1.0(6)***†	20.0 $\pm$ 2.6(7)
br/y(T)	76.9 $\pm$ 5.7(6)	85.3 $\pm$ 7.0(6)	69.0 $\pm$ 10.7(6)	19.4 $\pm$ 1.1(6)	31.9 $\pm$ 3.3(4)	21.6 $\pm$ 1.4(6)
blo+ /y	117.6 $\pm$ 14.6(6)*†	72.5 $\pm$ 9.4(5)	48.8 $\pm$ 18.4(3)	45.5 $\pm$ 3.1(6)***†	39.5 $\pm$ 1.7(4)	18.5 $\pm$ 1.5(4)
blo/y	70.9 $\pm$ 5.3(8)**†	69.4 $\pm$ 7.3(6)	47.3 $\pm$ 7.0(4)	21.7 $\pm$ 2.4(7)***†	31.5 $\pm$ 3.0(4)	17.3 $\pm$ 1.6(5)
blo/y(T)	105.8 $\pm$ 8.5(5)	82.1 $\pm$ 5.2(3)	52.0(2)	36.8 $\pm$ 1.6(5)	28.0 $\pm$ 1.0(4)	19.8 (2)
Cu+	138.0 $\pm$ 2.4(6)***†	94.3 $\pm$ 4.7(6)**	55.3 $\pm$ 2.0(6)***†	38.4 $\pm$ 1.6(6)***†	31.8 $\pm$ 1.5(6)***†	19.8 $\pm$ 1.0(6)***†
Cu-	41.4 $\pm$ 1.0(10)***†	60.0 $\pm$ 6.5(10)	43.4 $\pm$ 1.0(10)***†	13.6 $\pm$ 2.0(10)***†	18.8 $\pm$ 1.0(10)***†	14.0 $\pm$ 1.0(10)***†
Cu-(T)	92.4 $\pm$ 5.0(5)	57.6 $\pm$ 7.0(5)	52.6 $\pm$ 5.9(5)	45.9 $\pm$ 4.8(5)	28.5 $\pm$ 2.7(5)	20.5 $\pm$ 1.1(5)

† Significant difference in enzyme activity between untreated copper-replete and untreated mutant or Cu- mouse.
‡ Significant difference in enzyme activity between untreated and treated mice: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 3. Percentage of normal cytochrome *c* oxidase activity (Cyt.Ox.act.) and copper concentration in tissues of 13-day-old mice with a genetic or nutritional copper deficiency

	Concn. or activity (% of normal)					
	Condition . . . Br/y		Blo/y		Cu-	
	Cu	Cyt.Ox.act.	Cu	Cyt.Ox.act.	Cu	Cyt.Ox.act.
Heart	51	43.5	33	60	80	30.0
Liver	20	110.0	27	96	38	64.0
Kidney	355	99.8	282	97	62	78.5
Brain	28	27.5	38	48	72	35.4
Muscle	64	61.0	77	80	82	59.0
Lung	84	92.0	69	93	75	70.7

2. Nutritionally deficient mice. The 13-day-old Cu- mouse had significantly reduced cytochrome *c* oxidase activity in all tissues assayed (*P* < 0.002) (Table 2). The activity in brain, heart and skeletal muscle closely approximated that found in brindled mice.

Heart, brain and muscle enzyme activity were more severely affected than liver, kidney and lung activity, described in the Experimental section. The mice were in extrahepatic tissues (approx. 30%) (Table 3).

Effect of treatment. The copper-deficient mice and their respective copper-replete controls were injected at 7 days with a single subcutaneous injection of copper as described in the Experimental section. The mice were assayed for cytochrome *c* oxidase activity 24 h or 6 days after injection.

Cytochrome *c* oxidase levels were unchanged 24 h after injection (results not shown). At 13 days of age, cytochrome *c* oxidase activity in treated copper-replete mice remained identical with that in untreated controls.

In the 13-day-old treated brindled mutant, cytochrome *c* oxidase activity was increased significantly in brain (*P* < 0.001), heart (*P* = 0.001) and muscle (*P* < 0.001) with skeletal-muscle activity reaching normal levels (Table 2). Enzyme activity was unchanged in those tissues which had normal activity in untreated mutant mice.

Brain and heart cytochrome *c* oxidase activity was increased significantly in the blotchy mouse by copper injection, but there was no change in skeletal-muscle cytochrome *c* oxidase activity (Table 2).

All tissues of copper-injected nutritionally deficient mice had normal cytochrome *c* oxidase activity at 13 days, except liver, where the activity was not increased above the untreated level, and heart, where it was significantly increased (*P* < 0.001), but not to control activity values (Table 2).

Effect of temperature on enzyme activity. Cytochrome *c* oxidase activity of brindled-mouse and copper-replete-mouse liver and brain fractions increased with increasing assay temperature (Fig. 1). The temperature coefficient was 2.0 for brain and 2.7 for liver. SOD activity was unchanged over a temperature range of 23–37 °C (M. Phillips, unpublished work).

The rectal temperature of 13-day-old copper-deficient mice, both mutant and Cu- mice, was decreased by between 2 and 3 °C (M. Phillips, unpublished work). This hypothermia may affect enzyme activity *in vivo*.

SOD

Copper-replete mice. Tissue SOD activity was very similar in all genotypically normal copper-replete animals studied, that is, in both strains. There were

Fig. 1. Changes in cytochrome *c* oxidase activity with temperature

—, liver; ----, brain; ■, br/y; ●, blo/y; ▲, +/y.

significant differences between normal animals fed the purified diet supplemented with copper and those fed commercial mouse pellets (Table 4).

Copper-deficient mice. SOD activity was found to be significantly decreased in brindled-mutant brain ($0.01 < P < 0.02$) and heart ($0.02 < P < 0.05$) (Table 4). All other tissues had normal SOD activity.

In the blotchy mutant, only the brain had significantly decreased activity ($0.02 < P < 0.05$). The deficiency was less than that in the brindled mutant (Table 4).

Hepatic SOD was decreased ($P < 0.01$), in Cu— mice, but the decreases seen in other tissues were not statistically significant by parametric (*t* test) or non-parametric (Mann-Whitney) analysis (Table 4). Enzyme activity broadly reflected tissue copper concentration.

Effect of copper treatment. SOD activity was not increased in any tissue of 13-day-old mutants after copper injection at 7 days (Table 4). Activity was unchanged in tissues with normal levels in untreated mice (M. Phillips, unpublished work).

In copper-treated Cu—mice, SOD activity was significantly increased in liver ($P < 0.05$; Table 4).

DISCUSSION

In copper-deficient tissues of all genotypes cytochrome *c* oxidase activity was decreased to a greater extent than was SOD, except in the liver of Cu— mice, where the activity of both enzymes was decreased to the same extent. The deficiency of cytochrome *c* oxidase was not related to a decrease in the number of mitochondria present, since succinate dehydrogenase activity was normal in mutant tissues (M. Phillips, unpublished work).

The pattern of enzyme deficiency in the two age-matched mutants was strikingly similar, but the

Table 4. SOD activity in 13-day-old mice

Results are means $\pm$ S.E.M. (*n*) and were analysed by a two-tailed Student's *t* test. Abbreviations used: (T), treated animals; N.D., not determined.

Genotype	SOD (units/mg wet wt. of tissue)					
	Tissue . . . Liver	Kidney	Lung	Brain	Heart	Muscle
br ⁺ /y	25.6 $\pm$ 1.1(6)	7.8 $\pm$ 0.7(6)	6.4 $\pm$ 0.2(4)	3.6 $\pm$ 0.3(7)*†	3.8 $\pm$ 0.2(6)*†	2.0 $\pm$ 0.2(5)
br/y	25.1 $\pm$ 0.9(7)	7.4 $\pm$ 0.9(5)	6.5 $\pm$ 0.6(4)	2.6 $\pm$ 0.2(7)	3.1 $\pm$ 0.1(4)	2.2 $\pm$ 0.1(4)
br/y(T)	23.7 $\pm$ 1.0(4)	7.2(2)	N.D.	2.9 $\pm$ 0.4(6)	3.1 $\pm$ 0.5(4)	N.D.
blo ⁺ /y	25.5 $\pm$ 1.3(5)	7.4 $\pm$ 0.3(4)	6.8 $\pm$ 0.9(4)	4.3 $\pm$ 0.3(4)*†	3.5 $\pm$ 0.3(3)	1.5 $\pm$ 0.04(4)
blo/y	24.3 $\pm$ 0.8(7)	7.4 $\pm$ 1.1(4)	6.7 $\pm$ 0.7(5)	3.7 $\pm$ 0.1(6)	3.5 $\pm$ 0.4(3)	1.4 $\pm$ 0.09(5)
blo/y(T)	23.5 $\pm$ 1.3(5)	N.D.	N.D.	3.2 $\pm$ 0.2(3)	2.6 $\pm$ 0.1(3)	N.D.
Cu+	25.9 $\pm$ 1.4(6)**†	6.9 $\pm$ 0.7(3)	6.1 $\pm$ 0.3(3)	4.1 $\pm$ 0.04(4)	3.5 $\pm$ 0.3(5)	1.3 $\pm$ 0.1(5)
Cu—	18.0 $\pm$ 2.0(7)*†	5.9 $\pm$ 0.6(6)	5.6 $\pm$ 0.3(7)	3.6 $\pm$ 0.2(6)***†	3.9 $\pm$ 0.2(5)**†	1.5 $\pm$ 0.2(5)
Cu—(T)	22.7 $\pm$ 1.7(5)	6.3 $\pm$ 0.7(5)	6.0 $\pm$ 0.6(4)	4.7 $\pm$ 0.2(5)	5.2 $\pm$ 0.5(4)	1.7 $\pm$ 0.1(5)

† Significant differences in SOD activity between untreated copper-replete animals and untreated copper-deficient animals.

‡ Significant differences in SOD activity between untreated and treated copper-deficient animals: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

magnitude of the deficiency was greater in the more severely affected brindled mutants.

In the nutritionally deficient mouse the magnitude of the cytochrome *c* oxidase deficiency varied with the tissue studied, with brain having a much more severe deficiency than lung or kidney, despite the almost identical copper concentrations in these tissues. SOD activity was much more uniformly decreased.

The brain, heart and skeletal muscle of nutritionally deficient mice showed the greatest cytochrome *c* oxidase deficiency, as did these tissues in mutants. The presence of only a minor functional copper deficiency in the kidney and lung of copper-deprived normal mice suggests that attempts to prove tissue-specific expression of the genetic defect by analysis of copper concentration and enzyme activity will lead to error.

The normal activity of copper-dependent enzymes in mutant liver contrasts with the decreased enzyme activity in the liver of Cu— mice. Since hepatic copper concentration is similar in both genotypes, the copper must be contained in different pools.

The most interesting data came from comparisons of mice injected subcutaneously at 7 days with a copper load (50 µg). Cu+ sebaceous acid was shown to provide a biologically available source of copper in normal animals when administered subcutaneously.

In the nutritionally deficient mouse, both liver SOD and extrahepatic cytochrome *c* oxidase were returned to copper-replete levels by the copper injection. The failure of liver cytochrome *c* oxidase to respond to a treatment that increases liver copper concentration to more than three times normal suggests that the copper and enzyme are segregated intracellularly.

The mode of copper delivery may be important. Copper given orally will normally pass rapidly to the liver via the portal circulation, but it is likely that when given as a subcutaneous injection the element reaches peripheral tissues first.

Treating the mice with a greater dose of copper and oral administration of a 50 µg load should provide further information.

The response of cytochrome *c* oxidase activity to injection was identical in mutant and Cu— mice in brain and heart, and in brindled-mouse and Cu— mouse skeletal muscle. This is consistent with normal copper delivery to cytochrome *c* oxidase in these tissues in mutant mice when adequate copper is made available.

The cytochrome *c* oxidase activity in copper-injected brindled-mouse brain closely approximated that found in the brain of the untreated 13-day-old blotchy mouse. Neither treated brindled mutants nor blotchy mutants show clinical evidence of the brain abnormalities seen in the untreated brindled mutant (Danks, 1977; Mann *et al.*, 1979a; M. Phillips, unpublished work). It appears either that decreased cytochrome *c* oxidase activity accounts for the abnormal brain pathology in the brindled mutant or that increases in the activity of this enzyme to levels found in blotchy males is accompanied by an improvement in some other essential copper-requiring process in brindled brain.

It has been suggested that the magnitude of cytochrome *c* oxidase deficiency found in copper-deficient animals is insufficient to seriously disrupt oxidative phosphorylation (Gallagher *et al.*, 1956a,b; Smith *et al.*, 1976). However, as the body temperature of mutant and Cu— mice was lower than that of copper-replete mice, and as

cytochrome *c* oxidase activity increased with increasing temperature to 37 °C, measurements of cytochrome *c* oxidase activity *in vitro* made at a single temperature may significantly underestimate the deficiency *in vivo*. Assuming that it is possible to extrapolate the results obtained *in vitro* to the situation *in vivo*, brain cytochrome *c* oxidase activity in the brindled mutant is 20–25% of normal and not 33% as indicated by measurements *in vitro* at 25 °C (Table 3).

Although a 90% decrease in cytochrome *c* oxidase activity may be required before oxidative phosphorylation is significantly affected in adult rat liver (Gallagher *et al.*, 1956a,b), the effects of a lesser deficiency on brain during the critical brain growth period are unknown. It is suggested that cytochrome *c* oxidase deficiency cannot be dismissed as a possible cause of the morbidity in copper-deficient animals.

Hypothermia will also adversely affect enzymic processes not related directly to copper deficiency, but the disruption is likely to be most severe in tissues with a pronounced copper-dependent-enzyme deficiency.

In blotchy mice the skeletal-muscle cytochrome *c* oxidase remained at 'untreated' levels after injection. In the brindled mutant and in Cu— mice muscle cytochrome *c* oxidase increased to normal levels with treatment.

We (M. Phillips, J. Camakaris & D. M. Danks, unpublished work) found that recovery of radioactive copper from muscle of treated blotchy mice was approximately twice that recovered from muscle in treated Cu— mice. This suggests that the copper reaching this tissue is adequate for normal enzyme activity and that availability of copper is affected by the blotchy mutation.

It has been shown previously (Royce *et al.*, 1982) that the copper-dependent enzyme lysine oxidase does not respond to copper treatment in the skin of blotchy mutants, but that there is a good response to treatment in the brindled mutant. These differences in response to copper injection, together with the phenotypic differences between the two mutants, suggest that there is more than a quantitative difference between the two mutations, and may indicate that they are not allelic.

SOD activity was less affected than cytochrome *c* oxidase by nutritional or genetic copper deficiency in the tissues studied and was not increased in the mutants by copper injection.

The absence of any increase in SOD activity in the deficient tissues of mutant mice is interesting in view of the biological availability of injected copper to this enzyme implied by the results from Cu— mice. As the magnitude of cytochrome *c* oxidase deficiency is similar in Cu— mice and mutants, it is unlikely that competition for copper between these enzymes can explain the results.

Sequestration of copper on metallothionein in mutant tissues such as kidney may greatly decrease the length of time during which injected copper can be incorporated into enzymes. SOD is not deficient in mutant tissues at 7 days, the time of injection (M. Phillips, unpublished work). When required later in neonatal development it appears that copper is not biologically available. This could be tested by administering a second dose of copper, perhaps at 10 days.

Although the genetic defects in the brindled and blotchy mutants remain obscure, further comparative studies of cytochrome *c* oxidase and SOD in age-matched mutants, particularly when combined with a study of

nutritionally copper-deficient controls should greatly increase our understanding of the normal processes of copper homeostasis.

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