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Effect of Altered Copper Metabolism Induced by Mottled Alleles and Diet on Mouse Tyrosinase (40662)[1]

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In mice, alleles at the mottled locus on the X-chromosome produce dramatic influences on coat color (1, 2). Nonagouti mice homozygous for the wild-type (+) allele are black in color, whereas male mice hemizygous for tortoise (*To*), dappled (Mo^{dp}), brindled (Mo^{br}), viable brindled (Mo^{vbr}), or blotchy (Mo^{blo}) are gray (3). Females heterozygous for the mutant alleles are a mosaic of light and dark areas of coat color owing to random inactivation of the X-chromosome (3).

In addition to influences on pigmentation, alleles at the mottled locus are also known to influence behavior, longevity, activity of copper-containing enzymes, copper levels within various tissues, and development of connective tissues (4, 5). Collectively, observations to date on mottled mice suggest that the primary defect resulting in the complications of the "mottled syndrome," as in Menkes' kinky hair syndrome in man (6), is associated with faulty absorption, transport, or binding of copper (5, 7). The alleles at the mottled locus differ in their influences on the activity of copper-containing enzymes such as cytochrome c oxidase, lysyl oxidase, and ceruloplasmin (5, 8, 9). All mutant alleles, however, reduce melanin levels in the pelage, suggesting some common action on the copper-containing enzyme tyrosinase. The presence of coat color mosaicism in female mice heterozygous for mutant mottled alleles indicates that significant gene action occurs at the level of the melanocyte (4). Based on the observation that the intraperitoneal injection of copper results in darker hair color in brindled (Mo^{br}) male mice (10), it would appear that tyrosinase activity is increased when sufficient copper is administered in an appropriate fashion. As yet, however, no direct examination has been made of the form and function of tyrosinase within the melanocytes of mottled mice. The present study was carried out to determine the nature of action of alleles at the mottled locus on tyrosinase activity and the degree to which dietarily induced copper deficiency might mimic the genetic influence. Attention has been given not only to the effects of mottled alleles and dietarily induced copper deficiency on overall tyrosinase activity but also to those on the multiple forms of tyrosinase separable by polyacrylamide gel electrophoresis (PAGE). The latter observations were particularly instructive for the PAGE-separable varieties of tyrosinase are currently considered to represent stages in the developmental sequence leading to mature tyrosinase (11). Depending on genic constitution, a maximum of three soluble tyrosinases (T_1, T_{2-3}) are demonstrable in mouse "hair bulb" extracts subjected to PAGE (12).

Materials and methods. The following strains of mice were used: C57BL/6J, C57BL/6J-Mo^{blo}, C3H/HeJ-Mo^{br}, SWR/J, DBL/Wq, C57BL/Ha-*e*, PLN/Wq, PBR/St, LT/Ch, L/St, and Y/Wi. Hair growth was induced by plucking the quiescent (telogen) hairs from the dorsa of 8-week-old male and female mice. Hair bulbs were obtained on Day 10 of the ensuing hair growth cycle by methods fully described previously (13, 14). Hair bulbs were also obtained during the first spontaneous hair growth cycle from 7-day-old brindled C3H/HeJ-Mo^{br} males and their male agouti littermates.

Copper deficiency was induced in C57BL/6J black mice by placing weanlings on a copper-deficient diet (ICN Pharmaceuticals, Inc.). In some cases penicillamine (0.075%) and pyridoxine (0.02%) were added to the diet. The mice were maintained on the diet for approximately 4 weeks prior to plucking

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of the middorsal hair and for an additional 10 days thereafter. The period of treatment was sufficient to reduce the pigmentation from intense black to light brown in the hairs regrown. Hair bulbs were obtained on Day 10 of the hair cycle induced by plucking.

Each hair-bulb sample was homogenized in a Potter–Elvehjem all glass homogenizer in chilled 0.25 *M* sucrose (wet wt/vol, 0.1 g/ml), and centrifuged at 35,000*g* for 30 min at 0°. The resulting supernatant was subdivided with one aliquot being supplemented with copper by addition of 3×10^{-3} *M* $CuSO_4$ in 0.25 *M* sucrose (final concentration of $CuSO_4 = 3 \times 10^{-4}$ *M*) and the other diluted with the same volume of 0.25 *M* sucrose. The aliquots were then subjected to PAGE according to the method of Davis (15) with the following modifications: Distilled water was substituted for solution F, the sample gel was omitted, the enzyme preparation was layered directly on top of the spacer gel, and the bath buffer was 1/5th the stock concentration. The running time for the electrophoresis was 30–40 min at 3–5 mA/tube. Following electrophoresis, the gels were neutralized in 1.0 *M* phosphate buffer (pH 6.8) for 30 min. The multiple forms of tyrosinase were visualized by incubation of the gels in 0.1 *M* phosphate buffer (pH 6.8) containing 0.15% L-3,4-dihydroxyphenylalanine (dopa) for 2 hr at 37°. To preserve the tyrosinase patterns, gels were stored in 7.5% acetic acid. The gel electropherograms were assigned grades ranging from + to ++++ for color intensity of the dopa–melanin bands. The visual comparisons correlated well with objective densitometric measurements (14).

Some aliquots of the 35,000*g* supernatants were assayed for the tyrosine hydroxylating capacity of tyrosinase according to the method of Pomerantz (16). The reaction mixture contained 0.15 μmole L-tyrosine, 0.0025 mCi L-[3,5-^{3}H] tyrosine (New England Nuclear) and 0.15 μmole dopa in each 1.25 ml volume of 0.1 *M* phosphate buffer (pH 6.8). To 9 ml of this reaction mixture a 1-ml aliquot of the 35,000*g* supernatant was added; as in the PAGE, copper was either omitted or present in the supernatant in a concentration of 3×10^{-4} *M*. When determining the counts attributable to background, 0.25 *M* sucrose was substituted for the supernatant in the reaction vessel.

Results. Measurements by the Pomerantz radioassay with tyrosine as substrate revealed that the Mo^{br} allele in the hemizygous (Mo^{br}/y) and heterozygous ($Mo^{br}/+$) condition markedly reduced tyrosinase activity in hair-bulb supernatants containing soluble tyrosinase and particulate tyrosinase of the microsomal ("small granule") fraction (Table I). Addition of exogenous copper to the brindled hair-bulb supernatants greatly increased tyrosinase activity while having little effect on control samples (Table I). The tyrosinase activity of copper-supplemented hair-bulb supernatants of brindled mice never exceeded that of the copper-supplemented controls.

Polyacrylamide gel (dopa-incubated) electropherograms revealed that the mottled alleles, Mo^{br} and Mo^{blo}, markedly reduced the activity of the T_1 and T_{2-3} varieties of soluble tyrosinase although they had no effect on their mobilities (Fig. 1 and Table II). T_{2-3} designates a double band of activity, the discreteness of which remains uncertain (11, 12). The addition of copper to 35,000*g* supernatants of brindled and blotchy hair follicles resulted in a marked increase in T_1 activity but not of T_{2-3} judged by the intensity of melanin deposited in dopa-incubated electropherograms. In fact, T_{2-3} activity appeared to be reduced in these cases. No comparable effects were found with hair-bulb supernatants from C57BL/6J control mice (Fig. 1 and Table II). It is noteworthy that the T_1 activity of the copper-supplemented mottled

TABLE I. EFFECT OF COPPER SUPPLEMENTATION ON THE TYROSINASE ACTIVITY OF FOLLICULAR MELANOCYTES OF BLACK AND BRINDLED MICE

	cpm (^{3}HOH)[a]	
	Without copper	Copper added
Black ♀ (+/+) C57BL/6J adult control	2671	2940
Brindled ♀ ($Mo^{br}/+$) adult	230	1500
Brindled ♂ (Mo^{br}/y) 7 day old	0	316
Black Agouti ♂ (+/+) 7 day old control	781	779

[a] Pomerantz radioassay. Each sample consisted of 1 ml of supernatant from the pooled hair bulbs of 3–4 adult mice or 11–14 neonatal mice (w/v = 0.2 g/ml in adults and 0.1 g/ml in neonates). Experimental and control samples were run simultaneously. In each case a single sample was counted after 1 hr incubation.

supernatants decisively exceeded the T_1 activity of the copper-supplemented control supernatants.

The addition of copper to 35,000*g* hair-bulb supernatants from mice of a variety of coat colors resulting from action of genes independent of the mottled locus failed to increase tyrosinase activity as measured by the intensity of melanin synthesized within dopa-incubated electropherograms (Table III). In no case did exogenous copper result in the appearance of extra bands of tyrosinase or of tyrosinase bands where they were characteristically absent for a given genotype (17).

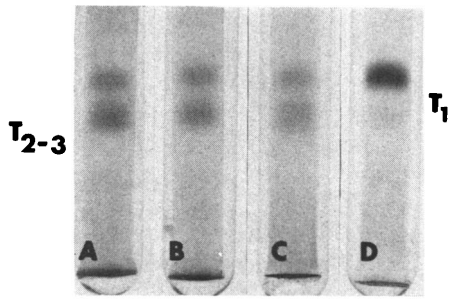

FIG. 1. Activity patterns of tyrosinase from follicular melanocytes of black (A and B) and brindled (C and D) female mice: A and C no copper supplementation; B and D copper added prior to electrophoresis.

The tyrosinase from C57BL/6J mice dietarily deprived of copper closely approximated that of the mottled mice in its properties. Tyrosinase activity as measured by the Pomerantz radioassay was markedly reduced in hair-bulb supernatants from mice with dietary copper deficiency and greatly increased when copper was added to the supernatants (Table IV). In addition, dopa-incubated electropherograms demonstrated that both the T_1 and T_{2-3} varieties of tyrosinase were reduced in activity by the copper deficiency and that the activity of T_1 but not of T_{2-3} could be increased by the addition of exogenous copper to the supernatants prior to electrophoresis (Table IV).

Discussion. The results of this study indicate that the uniform hypopigmentation of male mice hemizygous for mottled alleles and the patchy depigmentation of heterozygous females results from reduced tyrosinase activity within affected melanocytes. The reduced

TABLE II. EFFECT OF COPPER SUPPLEMENTATION *IN VITRO* ON THE ACTIVITY OF SOLUBLE TYROSINASES FROM MOTTLED AND BLACK MICE

	D-PAGE[a]			
	Without copper		Copper added	
	T_1	T_{2-3}	T_1	T_{2-3}
Black ♂, ♀ (+/+) C57BL/6J adult	+++	+++	+++	+++
Brindled ♀ (*Mo*^{br}/+) adult	+	+	++++	<+
Blotchy ♂ (*Mo*^{blo}/*y*) adult	+	<+	++++	<+

[a] D-PAGE = DOPA-incubated polyacrylamide gel electropherograms. <+ = perceptible but marginal activity.

TABLE III. EFFECT OF COAT COLOR GENES AND COPPER SUPPLEMENTATION *IN VITRO* ON THE ACTIVITY OF SOLUBLE TYROSINASES

Genotype							D-PAGE[a] relative activity with or without added copper	
a	b	c	d	e	ln	p	T_1	T_{2-3}
a/a	B/B	C/C	D/D	E/E	Ln/Ln	P/P	+++	+++
a/a		c/c					0	0
a/a			d/d				+++	+
a/a				e/e			++	0
a/a					ln/ln	p/p	++	0
a/a	b/b					p/p	+++	0
a/a	B^{lt}/B^{lt}						++	++
A^w/A^w		c^{ch}/c^{ch}					+++	+
A^y/A							+	0

[a] DOPA-incubated polyacrylamide gel electropherograms.

TABLE IV. EFFECT OF COPPER SUPPLEMENTATION *IN VITRO* ON THE TYROSINASE ACTIVITY OF HAIR-BULB SUPERNATANTS OF NORMAL AND COPPER-DEFICIENT C57BL/6J (BLACK) MICE

Treatment	PRA[a] cpm (^{3}HOH)		D-PAGE[b] relative tyrosinase activity			
	No copper	Copper added	No copper		Copper added	
			T_1	T_{2-3}	T_1	T_{2-3}
Complete diet	430	587	+++	+++	+++	+++
Copper-deficient diet	36	393	++	+	++++	+

[a] Pomerantz radioassay.
[b] Dopa-incubated electropherograms.

tyrosinase activity of mottled mice appears to derive substantially from restrictions in the amount of copper available for combination with the proteinaceous apoenzyme to produce functional tyrosinase. Dopa-incubated electropherograms show that exogenous copper enhanced soluble T_1 activity of mottled hair-bulb preparations to above control values. Tyrosinase activity toward tyrosine as a substrate is greatly increased when copper is added to mottled hair-bulb supernatants containing soluble tyrosinase and particle-bound microsomal tyrosinase, but its activity remains below that of the controls.

Collectively, the results suggest that in mottled mice considerable amounts of tyrosinase protein are synthesized but remain inactive owing to an inadequate supply of copper within the melanocyte. The essentially identical properties of mottled and dietarily induced copper-deficient tyrosinase preparations support this conclusion. The alleles of the mottled locus apparently influence tyrosinase activity and, as a consequence, pigmentation by acting on melanocytes to reduce copper levels below those necessary to produce a normal complement of functional tyrosinase molecules. The patchy light and dark color of female mice heterozygous for mottled alleles clearly indicates that the genes act at the level of the melanocytes. Applying the Lyon hypothesis (18), the dark areas are assumed to be populated by melanocytes with an active X-chromosome bearing the wild-type allele. These melanocytes are expected to produce more melanin than those in the light areas where the active X bears the mottled allele. In reality, it is possible that the normal melanocytes of at least some mottled heterozygotes synthesize somewhat less pigment than those of wild-type mice owing to impaired intestinal absorption, transport, or binding of copper. That this is the case is suggested by the increased pigmentation of hairs grown after intraperitoneal injections of copper in male brindled mice (10).

The electrophoretic studies reported here suggest that although T_1 and T_{2-3} are reduced owing to the action of the mottled alleles and of dietarily induced copper deficiency, only T_1 is increased in activity by copper supplementation. Apparently, the bulk of the copper-deficient apoenzyme is of the T_1 variety or converted to it by addition of copper. Evidence suggests that T_1 is a glycoprotein possibly derived from T_3 by addition of sialic acid and neutral sugars (11). It is not clear why T_3 should be saturated with copper whereas its putative derivative T_1 is not. Preliminary electron microscopic studies suggest that melanogenesis within individual melanosomes is reduced, indicating that they are deficient in tyrosinase or endowed with some copper-deficient tyrosinase.

At present it is not known whether T_1 tyrosinase apoenzyme migrates with the complete T_1 metalloprotein or is located at some other site in the gel. Future studies will isolate T_1 from the gels to determine whether addition of exogenous copper enhances its activity on reelectrophoresis and treatment with dopa reagent.

The present results suggest that the alleles of the mottled locus regulate melanogenesis by influencing the availability of copper at the level of the melanocyte perhaps by an action on copper transport or binding. In view of the multiple defects associated with the mottled alleles, it has been proposed that they represent a series of overlapping deletions with a common defect in pigmentation (9). A similar conclusion has been reached about the radiation-induced lethal alleles of the albino locus (19). Thus, the true nature of

the mottled locus remains to be resolved as does the simplicity or complexity of its primary action. Elucidation of this important point in mottled mice may help clarify the molecular genetics underlying the comparable condition of Menkes' kinky hair syndrome in humans (6, 20).

Summary. An analysis of tyrosinase activity in extracts of hair bulbs from mice bearing alleles at the mottled locus, Mo^{br} (brindled), and Mo^{blo} (blotchy), indicates that reduced melanogenesis in mottled mice is associated with restricted availability of copper. Although tyrosinase activity in 35,000*g* supernatants of hair-bulb homogenates of mottled mice is reduced compared to that of nonmottled controls, addition of copper to the former but not the latter supernatants markedly increases tyrosinase activity as measured in polyacrylamide gel electropherograms (dopa as substrate) or by the Pomerantz radioassay (tyrosine as substrate). Comparable results were obtained when tyrosinase from dietarily copper-deficient hypopigmented mice was assayed by either method. In the electropherograms of dietarily and genetically copper-deficient hair-bulb supernatants, the enhancement of activity is selectively restricted to the T_1 variety of tyrosinase. It is concluded that alleles at the mottled locus influence tyrosinase and consequently melanin pigmentation by acting on melanocytes to reduce copper levels below those necessary to produce a normal complement of functional tyrosinase molecules.

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