Copper metabolism in mottled mouse mutants

The effect of copper therapy on lysyl oxidase activity in brindled (*Mo^{br}*) mice

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Lysyl oxidase activity in extracts of skin from 10-day-old *Mo^{br}/Y* mice was found to be between 50 and 60% of that in corresponding extracts from littermate *+/Y* mice of the same age. It was increased to 84–150% of that in the latter by prior treatment of the *Mo^{br}/Y* mice at 7 days of age with a single subcutaneous injection of 50 µg of copper, retained as Cu⁺ in an alkyl polyether/sebacic acid solution. This suggests that in this form the copper is able to by-pass the block in copper metabolism and is deliverable to copper-requiring processes.

Mice bearing alleles at the X-linked locus mottled (*Mo*) have been proposed as an animal model of Menkes' syndrome in the human (Hunt, 1974; Danks, 1977). The most severely affected of these mutants surviving past birth are those bearing the allele 'brindled', hemizygous mutant males (*Mo^{br}/Y*) normally dying at around 14 days *post partum* (Mann *et al.*, 1980). Severe intestinal malabsorption of copper occurs in *Mo^{br}/Y* mice (Evans & Reis, 1978; Mann *et al.*, 1979a), and an abnormal distribution of copper among the tissues is apparent (Hunt, 1974; Camakaris *et al.*, 1979). Cultured fibroblasts both from patients with Menkes' syndrome and from *Mo^{br}/Y* mice sequester excessive amounts of copper (Goka *et al.*, 1976; Camakaris *et al.*, 1980; Sayed *et al.*, 1981), and one of us (Danks, 1977) has proposed that the fundamental defect in both cases might involve the increased affinity for copper of an intracellular copper-binding protein, such that intracellular copper is unavailable for copper-requiring enzymes, although other disturbances in copper metabolism may be invoked (Camakaris *et al.*, 1980). Treatment of Menkes' patients with copper salts or Cu–histidine, Cu–EDTA or Cu–albumin complexes administered intravenously or intramuscularly has been of little benefit (Danks, 1977; Garnica *et al.*, 1977; Daish *et al.*, 1978).

Abbreviations used: Cu⁺Seb(p), Cu⁺ in alkyl polyether/sebacic acid solution; NaCl/P_p, phosphate-buffered saline (0.1 M-NaH₂PO₄/0.15 M-NaCl, adjusted to pH 7.7 with NaOH).

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Treatment of *Mo^{br}/Y* mice at 7 days of age with a single subcutaneous injection of 50 µg of copper retained as Cu⁺ in an alkyl polyether/sebacic acid solution was previously observed to allow near normal growth and increased survival to at least 10 months of age (Mann *et al.*, 1979b). We report here the beneficial effect of such treatment on the activity of lysyl oxidase (no EC number has yet been assigned) in the skin of *Mo^{br}/Y* mice. The activity of lysyl oxidase, which initiates the cross-linking of collagen and elastin is copper-dependent and is decreased in copper-deficient tissues (Siegel, 1979). Determination of its activity in tissues thus provides an indication of the availability of copper to copper-dependent enzymes.

Experimental

Materials

Original breeding pairs of the *Mo^{br}* and *Mo^{blo}* strains were kindly supplied by Dr. D. M. Hunt, Queen Mary College, University of London, London E.1, U.K. Adult mice were housed in plastic boxes with steel tops and fed *ad libitum* on Mecon rat and mouse cubes (Gelman Clemco, Artarmon, N.S.W., Australia), which contained 16.7 µg of copper/g dry wt., and acidified water containing 2.5 µg of copper/ml.

Alkyl polyether was kindly supplied by I.C.I. (Australia) Ltd., Ascot Vale, Vic., Australia. It was diluted to 10% (v/v) with propane-1,2-diol. Cu⁺ (0.5%, w/v) as cuprous chloride and sebacic acid equimolar to copper were added as previously described (Mann *et al.*, 1981). L-[4,5-³H]Lysine

monohydrochloride (sp. radioactivity 75–100 Ci/mmol) was purchased from Amersham Australia, Sydney, N.S.W., Australia. A stock solution of 8 M-urea was deionized by passage through a mixed-bed ion-exchange resin (AG-501, X8, 20–50 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) before use. NaCl/P_i was passed through Chelex 100 resin (Bio-Rad). All glassware and dialysis tubing were soaked in 1% (w/v) EDTA and thoroughly washed with deionized water before use.

Methods

Treatment of mice. *Mo^{br}/+* mice were mated with normal male (+/Y) mice. *Mo^{br}/Y* mice and normal (+/Y) littermate mice were killed by decapitation at 10 days of age. The whole skins were removed, snap-frozen in liquid N₂ and stored at –20°C. In some litters *Mo^{br}/Y* mice were injected subcutaneously with 50 µg of copper in Cu⁺Seb(p) at 7 days of age, as described previously (Mann *et al.*, 1979b). Within each litter, skins from normal and mutant mice were pooled separately. In one experiment *Mo^{blo}/Y* and +/Y littermate mice were similarly used.

Lysyl oxidase assay. Lysyl oxidase activity in extracts of skin was determined by using an L-[4,5-³H]lysine-labelled collagen substrate prepared as described previously (Royce *et al.*, 1980). All extraction procedures were performed at 4°C. Pooled skins were homogenized in NaCl/P_i (5 ml/g wet wt. of tissue) by using a Polytron homogenizer. The homogenate was centrifuged (20000 g for 30 min) and the residue was re-extracted in NaCl/P_i and re-centrifuged. The residue was then extracted three times with 4 M-urea/0.05 M-Tris, pH 7.7, at 4°C (4.7 ml/g wet wt. of tissue), the first extraction being allowed to proceed for 1 h. The pooled NaCl/P_i extracts, the first urea extract and the pooled second and third urea extracts were filtered through glass wool and dialysed exhaustively overnight against NaCl/P_i. Lysyl oxidase activity in the extracts was determined by using (0.6–0.8) × 10⁶ c.p.m. of collagen substrate per reaction mixture as previously described (Royce *et al.*, 1980) except that an incubation period of 4 h was used. For each batch of substrate the assay system was characterized with extracts of skin from +/Y mice of 10 days of age to ensure that the volume of extracts assayed (0.1–0.2 ml NaCl/P_i extracts, 0.05–0.1 ml urea extracts) demonstrated activity in the linear region of the system. Lysyl oxidase activity was calculated as the amount of ³H₂O released from the substrate by the sample, with appropriate corrections (Royce *et al.*, 1980). Preliminary studies established that essentially all the lysyl oxidase activity was extracted from the skins under the conditions used, with only about 20% of the activity being in the NaCl/P_i extracts. Total lysyl oxidase

activity in the skins was obtained by the summation of the activity in each of the extracts. The protein content of the extracts was determined by a modification of the method of Lowry *et al.* (1951) as described previously (Royce *et al.*, 1980).

Results and discussion

Lysyl oxidase activity in extracts of skin from 10-day-old *Mo^{br}/Y* mice was 50–60% of that in extracts of skin from littermate +/Y mice, as shown in Table 1. This finding supports the hypothesis of Danks (1977) that a functional copper deficiency exists in which intracellular copper is unavailable for copper-requiring processes. Similarly reduced levels of lysyl oxidase activity in the skin of *Mo^{br}/Y* mice have been reported by Rowe *et al.* (1977). Despite this, *Mo^{br}/Y* mice die at about 14 days *post partum*, exhibiting signs of a neurological disorder (Hunt, 1974) without evidence of aortic aneurysms or other connective-tissue defects (Rowe *et al.*, 1974), and it

Table 1. Lysyl oxidase activity in extracts of skin from untreated and treated *Mo^{br}/Y* and *Mo^{blo}/Y* mice

Lysyl oxidase activity was determined in extracts of the pooled skins of 10-day-old *Mo^{br}/Y* and littermate +/Y mice as described in the text. In certain litters the *Mo^{br}/Y* mice were treated at 7 days of age with a subcutaneous injection of 50 µg of copper as Cu⁺Seb(p). Lysyl oxidase activity in the extracts was calculated as the amount of ³H determined as c.p.m. of ³H₂O released from the [³H]lysine-labelled collagen substrate by the extracts, with appropriate corrections. The number of *Mo^{br}/Y* and +/Y mice in each litter is indicated in parentheses. For litters 10 and 11, *Mo^{blo}/Y* and littermate +/Y mice were similarly processed.

Litter	Lysyl oxidase activity (c.p.m./mg of protein)		$\left(\frac{Mo^{br}/Y}{+/Y}\right) \times 100$
	+/Y	<i>Mo^{br}/Y</i>	
Untreated			
1	896 (2)	487 (2)	55.5
2	982 (1)	600 (1)	61.1
3	1466 (1)	840 (1)	57.3
4	871 (2)	529 (2)	60.8
5	1165 (2)	601 (2)	51.7
Treated			
6	572 (1)	860 (1)	150
7	755 (1)	634 (1)	84
8	754 (2)	984 (2)	131
9	1183 (1)	1663 (1)	141
Untreated	+/Y	<i>Mo^{blo}/Y</i>	$\left(\frac{Mo^{blo}/Y}{+/Y}\right) \times 100$
10	1350 (2)	299 (2)	22.1
Treated			
11	1044 (1)	369 (1)	35.3

may be that enzyme levels greater than 50% of normal are sufficient to maintain the integrity of the connective tissues. Prior administration to 7-day-old *Mo^{br}/Y* mice, by subcutaneous injection, of 50 µg of copper as Cu⁺Seb(p) resulted in a dramatic elevation of lysyl oxidase activity as shown in Table 1, suggesting that in this form the copper is available to copper-requiring enzymes. This may be related to the provision of copper as Cu⁺ (Mann *et al.*, 1979b). Although the possibility of a deletion-type mutation primarily affecting lysyl oxidase as suggested by Rowe *et al.* (1977) cannot be ruled out, such a defect would be unlikely to respond to copper therapy. There is also strong evidence for the involvement of copper in regulation of the synthesis of lysyl oxidase (Rayton & Harris, 1979).

Mice bearing the allele 'blotchy' at the 'mottled' locus (*Mo^{blo}/Y*), although apparently less severely affected than *Mo^{br}/Y* mice in that they survive to about 150 days of age, have been reported to show only about 30% of normal lysyl oxidase activity in skin (Rowe *et al.*, 1977) and die from aortic aneurysm (Rowe *et al.*, 1974). In a preliminary experiment we have confirmed this result (Table 1) and observed that *Mo^{blo}/Y* mice appear to be less responsive to copper therapy than do *Mo^{br}/Y* mice (Table 1). The reason for the differences between the two mutants is not clear. Other studies on *Mo^{blo}/Y* mice have shown that these mutants fail to show a return to normal growth after single-dose treatment with Cu⁺Seb(p) (Mann *et al.*, 1981).

The beneficial effects of treatment of *Mo^{br}/Y* mice with Cu⁺Seb(p) suggest new lines of therapy for Menkes' syndrome.

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