Copper metabolism in mottled mouse (*Mus musculus*) mutants

Studies of blotchy (*Mo^{blo}*) mice and a comparison with brindled (*Mo^{br}*) mice

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1. Copper concentrations were low in many organs of *Mo^{blo}/Y* mice, but very high in the gut. Absorption of ⁶⁴Cu was seen to be very low when related to the absorption of cyano[⁵⁷Co]cobalamin. The results in *Mo^{blo}/+* mice were intermediate. 2. Copper therapy temporarily ameliorated many effects of the mutation in *Mo^{blo}/Y* mice, but did not improve the rate of weight gain as has been achieved previously in *Mo^{br}/Y* mice. Lower capacity for a 'depot dose' effect at the site of injection may explain the difference. 3. The distribution of ⁶⁴Cu after administration into the bloodstream of *Mo^{blo}/Y* mice altered from an initially normal state to one that resembled the abnormal distribution of pre-existing copper by 48 h. This indicated that the later mechanisms of copper distribution were at fault. *Mo^{blo}/+* mice were equally affected. 4. The alteration of copper homeostasis in blotchy mice was similar to that observed in brindled mice previously and in the present studies, although generally less severe. This is consistent with allelism of the two mutations.

A series of X-linked mutations appear to be allelic at the locus mottled (*Mo*) in the house mouse (*Mus musculus*). However, allelism cannot be proved because of X-chromosome inactivation. Affected mice have a defect of copper transport that results in severe copper deficiency and a wide variety of consequent effects (Hunt, 1974; Danks, 1977). They provide a model of human Menkes' syndrome (Hunt, 1974), and the remarkable similarity suggests homology with the human mutation (Danks, 1977). Females heterozygous for the different mutations, e.g. *Mo^{blo}/+*, are viable and fertile. However, the severity of the effects varies between the different mutations in hemizygous mutant males. Brindled males, *Mo^{br}/Y*, are the most affected of those that survive past birth, and they die at around 14 days of age (Fraser *et al.*, 1953). Blotchy males, *Mo^{blo}/Y* (Russell, 1960), are the least affected of the series and survive to around 6 months of age (Andrews *et al.*, 1975).

Studies with suckling brindled mice have shown that very low copper concentrations are apparent in many organs of *Mo^{br}/Y* mice (Hunt, 1974; Camakaris *et al.*, 1979; Mann *et al.*, 1979a), due to a severe malabsorption of copper by the small

intestine (Hunt, 1974; Evans & Reis, 1978; Mann *et al.*, 1979b). Copper concentrates down the length of the small intestine, and very high concentrations of copper are present in ileal epithelial cells and luminal contents (Camakaris *et al.*, 1979). Similarly, defective placental transfer of copper to *Mo^{br}/Y* fetuses results in copper deficiency of the mutant foetus and a high placental copper concentration (Camakaris *et al.*, 1979; Mann *et al.*, 1980). Kidney also has an abnormally high concentration of copper, probably because of its role in copper re-absorption (Hunt, 1976; Camakaris *et al.*, 1979). The high concentrations of copper in mutant gut, placenta and kidney parallels the excessive accumulation of copper in isolated cultured cells of *Mo^{br}/Y* mice (Camakaris *et al.*, 1980). Furthermore, many other organs of *Mo^{br}/Y* mice accumulated excessive amounts of ⁶⁴Cu when this was administered by intracardiac injection in order to by-pass the intestinal barrier to copper transport. This indicates that the expression of the mutation is not confined to those organs involved in copper absorption, transfer and re-absorption (Mann *et al.*, 1979b). Copper therapy has permitted fertility and long-term survival of *Mo^{br}/Y* mice, the greatest response in the rate of

weight gain being achieved with a cuprous chloride/alkyl polyether/sebacic acid solution (Mann *et al.*, 1979a).

Of the other mutations of the mottled series, some studies of copper metabolism have been performed with blotchy mice (Hunt, 1976; Starcher *et al.*, 1978). However, the observations are limited, and the older ages of the mice used do not permit direct comparison with the studies of suckling brindled mice. The present paper reports findings for blotchy mice obtained by using methods similar to those previously employed for brindled mice of the same age (Camakaris *et al.*, 1979; Mann *et al.*, 1979a,b). Some further studies of brindled mice have also been performed.

Experimental

Materials

Original breeding pairs of our blotchy and brindled strains were kindly supplied by Dr. David Hunt, Department of Plant Biology and Microbiology, Queen Mary College, University of London, London E1 4NS, U.K. All blotchy mice used in the present studies were obtained from matings between heterozygous female ($Mo^{blo}/+$) mice and normal male (+, Y) mice, which would be expected to produce equal proportions of +/Y, normal female (+/+), mutant male (Mo^{blo}/Y) and $Mo^{blo}/+$ mice. Brindled mice were obtained by the same method of mating.

Adult mice were fed *ad libitum* on Mecon rat and mouse cubes (NAFAG formulation) [Bunge (Australia) Pty. Ltd., Melbourne, 3000, Vic. Australia], which contained $11.8 \pm 4.7 \mu\text{g}$ of copper/g, and acidifier water containing $0.27 \pm 0.02 \mu\text{g}$ of copper/ml.

$^{64}\text{CuCl}_2$ (half-life 12.8 h) was purchased from the Australian Atomic Energy Commission, Lucas Heights, 2234, N.S.W., Australia, and cyano[^{57}Co]cobalamin (vitamin B-12) (half-life 270 days), from Amersham Australia Pty. Ltd., Sydney, 2000, N.S.W., Australia. The radioactivity of samples was measured with an LKB gamma counter.

Copper concentrations were determined with a Varian-Techtron CRA-90 atomic-absorption spectrophotometer incorporating a BC-6 background-corrector module. All glassware was washed in concentrated HNO_3 , and surgical instruments were washed in EDTA as described by Stevens (1972).

The copper solutions used for the copper-therapy experiments consisted of 0.5% (w/v) Cu^+ or Cu^{2+} as copper chloride, sebacic acid equimolar to copper, 10% (v/v) alkyl polyether and 90% (v/v) propane-1,2-diol. These solutions are hereafter termed $\text{Cu}^+\text{Seb}(\text{p})$ and $\text{Cu}^{2+}\text{Seb}(\text{p})$ (Mann *et al.*, 1979a).

All injections into suckling mice were made with

10- μl SGE micro-syringes (Scientific Glass Engineering Pty. Ltd., North Melbourne, 3051, Vic. Australia).

Methods

Assays. Copper concentrations in acid digests of whole organs, small-intestinal epithelial cells [obtained by digestion with collagenase and hyaluronidase (Camakaris *et al.*, 1979)] and urine, urine creatinine concentration (by the Jaffé reaction), haemoglobin concentration (by the cyanomethaemoglobin method) and caeruloplasmin oxidase activity (by oxidation of *p*-phenylenediamine) were determined as previously described (Stevens, 1972; Camakaris *et al.*, 1979).

Intestinal absorption of $^{64}\text{CuCl}_2$ and cyano[^{57}Co]cobalamin. This experiment was conducted on both blotchy and brindled mice. $^{64}\text{CuCl}_2$ and cyano[^{57}Co]cobalamin were administered together by injection into the tail vein of lactating mothers, which allowed the suckling mice to receive radioactively labelled milk. This method was more physiological than the injections of ^{64}Cu directly into the intestines of neonatal mice that were used previously (Mann *et al.*, 1979b).

Cyano[^{57}Co]cobalamin was employed as a reference of the amount of milk that each mouse consumed. The validity of this approach is demonstrated in the Results section.

The experiments were conducted on groups of mice each comprising two +/Y, two Mo^{blo}/Y and two $Mo^{blo}/+$ suckling mice, and each fed by a different $Mo^{blo}/+$ mother. This was achieved by re-arranging the litters 3 days before experimentation. The same procedure was used for brindled mice.

When the litters were between 6 and 9 days of age, the radioisotopes were administered to the mothers. After the desired length of time, the suckling mice were removed and anaesthetized with diethyl ether. The main veins of the neck were exposed by removing the surrounding skin, and then cut so that blood could be collected with a Pasteur pipette. The whole of the alimentary tract was removed, and ^{64}Cu radioactivity was measured in the collected blood and the whole of the remaining carcass. ^{57}Co radioactivity did not interfere with the measurements. Dissecting instruments were rinsed in 1% (w/v) EDTA and dissection paper was changed after each dissection to avoid cross-contamination. After the sample of highest ^{64}Cu radioactivity had decayed to below background values, approx. 3 weeks, ^{57}Co radioactivity of the samples was measured.

Copper therapy. The methods used for copper therapy of blotchy mice in these experiments were the same as those previously used for brindled mice (Mann *et al.*, 1979a).

+/Y and Mo^{blo}/Y mice were used. Mice of 7 days

of age received a single subcutaneous injection of 50 µg of copper as either Cu⁺Seb(p) or Cu²⁺Seb(p) in the dorsal midline. The mice were weighed at 7 days and then every 3 days up to 61 days of age. Litters were kept at four to five mice to decrease differences in growth due to differing litter size. Each litter contained at least two +/Y and two *Mo^{blo}/Y* mice. At least one +/Y and one *Mo^{blo}/Y* mouse remained untreated as a control. Tissue copper concentrations and caeruloplasmin oxidase activities were determined at 3 days and 54 days after injection.

Distribution of ⁶⁴Cu after intracardiac injection. +/Y, *Mo^{blo}/Y* and *Mo^{blo}/+* mice of 9–12 days of age were used. ⁶⁴Cu was administered directly into the bloodstream by intracardiac injection. The procedures of intracardiac injection, and subsequent handling and dissection of the mice, were the same as those described by Mann *et al.* (1979b). During the prescribed time interval, mice were kept in waxed paper cups that had a layer of filter paper covered by gauze at the bottom to collect urine and to separate faeces. ⁶⁴Cu radioactivity was measured in the collected blood, urine and faeces, and the whole of the kidneys, skin, brain, liver, gut (plus luminal contents), spleen, lungs, heart, and the remaining carcass.

To obtain an estimate of the amount of radioisotope excreted in the bile, the intestine was ligated at the upper jejunal loop in some other animals immediately before intracardiac injection. The isolated stomach and duodenum then served as a reservoir for bile. ⁶⁴Cu radioactivity was measured in the same organs as in non-ligated mice, except that the stomach and duodenum and the remainder of the gut were measured separately.

Results

Assays

Malabsorption of copper was indicated in *Mo^{blo}/Y* and *Mo^{blo}/+* mice by large amounts of copper present in the whole gut (plus luminal contents) and copper deficiency in the rest of the body. [Amount of copper (µg) in +/Y gut, 1.00 ± 0.18; rest of body, 11.37 ± 2.21; *Mo^{blo}/Y* gut, 3.73 ± 1.40; rest of body, 5.05 ± 1.60; *Mo^{blo}/+* gut, 2.18 ± 0.53; rest of body, 7.13 ± 1.71. Four groups of one each of +/Y, *Mo^{blo}/Y* and *Mo^{blo}/+* littermates were used. Results are means ± s.d. Student's *t* test of copper in gut and rest of body respectively; +/Y versus *Mo^{blo}/Y*, *P* < 0.01, *P* < 0.01; +/Y versus *Mo^{blo}/+*, *P* < 0.01, *P* < 0.05; *Mo^{blo}/Y* versus *Mo^{blo}/+*, *P* > 0.05, *P* > 0.05]. The copper concentrations in the distal small intestine were extremely high in *Mo^{blo}/Y* mice, as shown in ileal epithelial cells and luminal contents [Table 1, part (ii)]. Copper concentrations were also high in whole gut (plus luminal contents) and kidney,

not significantly different in lung, skin, carcass and testis, low in brain, spleen, heart, thigh muscle, plasma and erythrocytes, and extremely low in liver [Table 1, parts (i)(a) and (ii)]. Copper concentrations in *Mo^{blo}/+* mice were intermediate in all cases, except in kidney and urine, where concentrations were even higher than in *Mo^{blo}/Y* mice [Table 1, parts (i)(a) and (ii)]. Caeruloplasmin oxidase activity was also low in *Mo^{blo}/Y* and *Mo^{blo}/+* mice; however, haemoglobin concentrations were normal [Table 1, part (ii)].

The total copper in the various organs of *Mo^{blo}/Y* and *Mo^{blo}/+* mice was expressed as a percentage of the total body copper (excluding that in the whole gut and luminal contents) and compared with +/Y mice. The percentage in kidney, lung, heart, skin and carcass was higher in the mutants, whereas that in the liver was lower. No differences from +/Y mice were apparent in the percentage of copper in brain and spleen. The pattern in *Mo^{blo}/+* mice was as abnormal as in *Mo^{blo}/Y* mice [Table 1, part (i) (b)].

To compare with the copper concentrations in gut and ileal epithelial cells of blotchy mice (Table 1), copper concentrations of these were determined in brindled mice of 9–11 days of age. [Whole gut (plus luminal contents): eight groups of one each of +/Y, *Mo^{br}/Y* and *Mo^{br}/+* littermates were used. Results are means ± s.d. for the amount of copper (µg/g dry wt.): +/Y, 15.3 ± 6.3; *Mo^{br}/Y*, 88.3 ± 35.5; *Mo^{br}/+*, 57.3 ± 40.3. Student's *t* test: +/Y versus *Mo^{br}/Y*, *P* < 0.01; +/Y versus *Mo^{br}/+*, *P* < 0.05; *Mo^{br}/Y* versus *Mo^{br}/+*, *P* > 0.05. Ileal epithelial cells: six groups of one each of +/Y and *Mo^{br}/Y* littermates were used. Results are means ± s.d. for the amount (µg) of copper/10⁹ cells: +/Y, 28.28 ± 19.7; *Mo^{br}/Y*, 243.4 ± 125.89. Student's *t* test: *P* < 0.01].

Intestinal absorption of ⁶⁴CuCl₂ and cyano[⁵⁷Co]cobalamin

The validity of using cyano[⁵⁷Co]cobalamin as a reference of the amount of milk that each mouse consumed depended on its normal utilization by the suckling mutant and heterozygous mice. This was indicated in the following observations. *Mo^{blo}/Y*, *Mo^{blo}/+*, *Mo^{br}/Y* and *Mo^{br}/+* mice all absorbed similar amounts of ⁵⁷Co at 7 and 24 h (Table 2a). Furthermore, the distribution of ⁵⁷Co among various organs was found to be the same in both +/Y and *Mo^{br}/Y* mice at 10 min, 7 and 24 h after intracardiac injection. Only approx. 20% of the administered dose was excreted in both +/Y and *Mo^{br}/Y* mice over the 24 h (J. R. Mann, unpublished work).

The amount of ⁶⁴Cu present in all mice at 7 and 24 h after administration (Table 2a) would represent virtually the total amount of ⁶⁴Cu absorbed over these time intervals. This is apparent from other

Table 1. Copper concentrations and distribution, caeruloplasmin oxidase activity and haemoglobin concentration in 9–11-day-old blotchy mice

(i) (a) Copper concentrations (μg of copper/g dry wt.) were measured by using the whole of the different organs and the remaining carcass of each mouse. Results are means $\pm$ S.D. This allowed calculation of (b) percentages of copper in the various organs with respect to total body copper excluding copper in the whole gut (plus luminal contents). Four groups of one each of +/Y, Mo^{blo}/Y and $Mo^{blo}/+$ littermates were used. Results are means $\pm$ S.D. Student's *t* test was used to assess significance of values in (a). +/Y versus Mo^{blo}/Y or $Mo^{blo}/+$: * $P < 0.05$; ** $P < 0.01$; † not significant. (ii) These assays were performed on mice different from those in (i). Copper concentrations are in $\mu\text{g}/\text{g}$ dry wt. in thigh muscle, testis and ileal luminal contents, $\mu\text{g}/10^9$ cells in ileal epithelial cells, $\mu\text{g}/100\text{ ml}$ in plasma and erythrocytes, and $\mu\text{g}/\text{mmol}$ of creatinine in urine. Haemoglobin concentration is in $\mu\text{g}/100\text{ ml}$ and caeruloplasmin oxidase activity in A_{230} units. Results are means $\pm$ S.D. for four observations, unless otherwise stated in parentheses. The range of observations is also given for ileal epithelial cells and luminal contents. Caeruloplasmin oxidase activity was determined on the pooled plasma of four mice. Student's *t* test was used to assess significance, and is indicated as in (i). Abbreviation used: ND, not determined.

	+/Y		Mo^{blo}/Y		$Mo^{blo}/+$		Significance Mo^{blo}/Y versus $Mo^{blo}/+$
	(a)	(b)	(a)	(b)	(a)	(b)	
(i) Whole gut	14.8 $\pm$ 2.7	—	69.3 $\pm$ 22.2**	—	31.5 $\pm$ 8.2**	—	*
Brain	7.5 $\pm$ 1.8	2.86	2.4 $\pm$ 0.2**	2.10	4.0 $\pm$ 0.8*	2.43	**
Liver	113.7 $\pm$ 49.8	43.57	9.4 $\pm$ 1.3**	6.15	13.4 $\pm$ 5.5**	7.51	†
Kidney	11.6 $\pm$ 0.8	1.50	25.6 $\pm$ 1.1**	5.83	39.0 $\pm$ 22.3*	7.54	†
Spleen	9.6 $\pm$ 2.7	0.50	3.6 $\pm$ 1.4**	0.24	4.9 $\pm$ 0.7*	0.37	†
Lung	6.3 $\pm$ 0.9	0.95	4.9 $\pm$ 1.4†	1.38	5.5 $\pm$ 1.4†	1.29	†
Heart	19.2 $\pm$ 3.3	1.06	11.4 $\pm$ 2.6**	1.15	15.7 $\pm$ 2.6†	1.35	†
Skin	4.1 $\pm$ 1.0	20.40	3.0 $\pm$ 1.2†	22.35	4.0 $\pm$ 1.4†	26.89	†
Carcass	5.3 $\pm$ 3.0	29.15	5.7 $\pm$ 1.8†	60.77	5.6 $\pm$ 2.0†	52.61	†
(ii) Thigh muscle	4.3 $\pm$ 0.7	—	2.6 $\pm$ 0.5**	—	3.6 $\pm$ 0.6†	—	*
Testis	6.8 $\pm$ 0.7	—	5.6 $\pm$ 1.1†	—	—	—	—
Ileal epithelial cells	35.7 $\pm$ 19.8 (6) (17.6–62.9)	—	356.4 $\pm$ 183.9 (6) (83.3–516.4)**	—	ND	—	—
Ileal luminal contents	43.8 $\pm$ 14.4 (3) (35.2–60.4)	—	658.2 $\pm$ 479.4 (3) (299.3–1202.7)†	—	ND	—	—
Plasma	67.8 $\pm$ 11.6	—	36.0 $\pm$ 9.9**	—	50.2 $\pm$ 7.3*	—	†
Erythrocytes	146.2 $\pm$ 27.7	—	66.5 $\pm$ 5.2**	—	87.7 $\pm$ 8.4**	—	**
Urine	70.4 $\pm$ 24.7	—	75.5 $\pm$ 19.1†	—	148.4 $\pm$ 43.5**	—	**
Caeruloplasmin	0.477	—	0.165	—	0.389	—	—
Haemoglobin	10.1 $\pm$ 0.8	—	10.5 $\pm$ 0.5†	—	10.0 $\pm$ 0.9†	—	†

Table 2. Absorption of $^{64}\text{CuCl}_2$ and cyano ^{57}Co cobalamin in blotchy and brindled mice

Heterozygous mothers suckling mice 6–9 days of age received intravenously 300 μl of a solution containing 188–234 μCi of ^{64}Cu (30 μg of total copper as CuCl_2), and 0.25 μCi of ^{57}Co (0.25 μg of total cyanocobalamin). Suckling mice from all litters were killed at 7 and 24 h after administration of radioisotope. (a) Percentage of the administered dose recovered in the whole suckling mouse except gut (absorbed portion of the dose). Results are means $\pm$ S.D., with the number of observations in parentheses. (b) The amount of ^{64}Cu absorbed divided by the amount of ^{57}Co absorbed obtained by using values in (a), expressed as a percentage of the quotient obtained for +/Y mice.

	Blotchy mice			Brindled mice		
	+/Y	Mo^{blo}/Y	$Mo^{blo}/+$	+/Y	Mo^{br}/Y	$Mo^{br}/+$
(a) 7 h						
^{57}Co	2.32 $\pm$ 0.53 (4)	1.59 $\pm$ 0.08 (4)	2.56 $\pm$ 0.57 (4)	2.17 $\pm$ 1.13 (4)	2.70 $\pm$ 1.48 (4)	2.42 $\pm$ 1.73 (3)
^{64}Cu	0.91 $\pm$ 0.16 (4)	0.06 $\pm$ 0.01 (4)	0.38 $\pm$ 0.17 (4)	0.61 $\pm$ 0.26 (4)	0.05 $\pm$ 0.03 (4)	0.42 $\pm$ 0.32 (3)
24 h						
^{57}Co	13.16 $\pm$ 0.79 (4)	10.43 $\pm$ 2.31 (4)	14.59 $\pm$ 1.33 (2)	11.84 $\pm$ 2.67 (4)	9.07 $\pm$ 3.46 (4)	12.97 $\pm$ 1.41 (3)
^{64}Cu	3.52 $\pm$ 0.44 (4)	0.55 $\pm$ 0.01 (4)	1.44 $\pm$ 0.57 (2)	2.75 $\pm$ 0.49 (4)	0.27 $\pm$ 0.11 (4)	1.27 $\pm$ 0.34 (3)
(b) $^{64}\text{Cu}/^{57}\text{Co}$						
7 h	100	10	38	100	7	62
24 h	100	20	37	100	13	43

studies of suckling mice, in which the recovery of the administered dose of ^{64}Cu at 48 h after intracardiac injection showed excretion of copper to be negligible.

[Blotchy mice 9–11 days of age: +/Y, 98.9%; Mo^{blo}/Y , 104.2%, $Mo^{blo}/+$, 100.2%, in the present experiments. Brindled mice 10–12 days of age: +/Y,

103.2%; Mo^{br}/Y , 100.7% $Mo^{br}/+$, 106.6%, in the experiments of Mann *et al.*, 1979b). Percentages are the means of two observations.]

The amount of ^{64}Cu absorbed over 7 and 24 h was very low in both Mo^{blo}/Y and Mo^{br}/Y mice in comparison with $+/Y$ mice, whereas absorption by $Mo^{blo}/+$ and $Mo^{br}/+$ mice was intermediate (Table 2a). It was evident that Mo^{blo}/Y mice absorbed more ^{64}Cu (corrected for absorption of ^{57}Co) than Mo^{br}/Y mice over 7 and 24 h (Table 2b). $Mo^{blo}/+$ mice absorbed relatively less ^{64}Cu than $Mo^{br}/+$ mice over 7 and 24 h, although the difference at 24 h was not large (Table 2b). The amount of ^{64}Cu absorbed in Mo^{blo}/Y and Mo^{br}/Y mice in relation to that absorbed in $+/Y$ mice, doubled between 7 and 24 h (Table 2b). This is consistent with other observations indicating that copper can be absorbed in mutant mice by pinocytosis at the distal small intestine (Mann *et al.*, 1979b).

Copper therapy

Within 24 h after subcutaneous injection of the $Cu^{+}Seb(p)$ and $Cu^{2+}Seb(p)$ solutions, pigmentation had appeared in the skin of Mo^{blo}/Y mice. The kinky whiskers straightened over about 20 days, but by 61 days of age had again become kinky.

At 3 days after injection of $Cu^{+}Seb(p)$, the copper concentrations were slightly increased in most tissues of treated $+/Y$ mice, and greatly increased in liver and skin (Table 3). Most of the copper present in the skin of treated $+/Y$ and Mo^{blo}/Y mice probably represented the residue of the injected dose.

In treated Mo^{blo}/Y mice, copper concentrations had been increased to at least those of untreated $+/Y$ mice, except in brain (Table 3). However, it is apparent that the distribution of copper was still abnormal. Kidney and testis accumulated copper well above the concentrations in $+/Y$ mice, whereas the liver concentrations fell well short of that seen in treated $+/Y$ mice.

Despite the increase in tissue copper concentrations, no improvement in the rate of weight gain was achieved up to 61 days of age with either $Cu^{+}Seb(p)$ or $Cu^{2+}Seb(p)$ in treated Mo^{blo}/Y mice (Fig. 1). Similarly, no significant change was seen in $+/Y$ mice (Fig. 1).

By 54 days after injection, most organs of treated Mo^{blo}/Y mice were again copper-deficient, concentrations being very similar to those of untreated Mo^{blo}/Y mice (Table 3). Caeruloplasmin oxidase activity was also low (Table 3). However, these deficiencies were not as severe as those in untreated Mo^{blo}/Y mice of 10 days of age. The copper concentration in kidney was still high (Table 3).

Distribution of ^{64}Cu after intracardiac injection

The distribution of ^{64}Cu with time was greatly altered in Mo^{blo}/Y and $Mo^{blo}/+$ mice. The results for some organs are shown in Figs. 2(a)–2(d) and for all organs measured in Table 4. The alterations in $Mo^{blo}/+$ mice were similar to those in Mo^{blo}/Y mice. At 10 min after injection, uptake of ^{64}Cu into the various organs was similar in $+/Y$, Mo^{blo}/Y and $Mo^{blo}/+$ mice, but differences became apparent

Table 3. Copper concentrations of tissues and caeruloplasmin oxidase activity in blotchy mice 3 and 54 days after copper therapy

All mice were treated with $Cu^{+}Seb(p)$. Data for untreated mice at 3 days after injection are taken from Table 1. Caeruloplasmin oxidase activity (A_{230} units) was measured in a pooled sample of four mice for mice 3 days after injection, and individually for mice 54 days after injection. The means of two observations are expressed as μg of copper/g dry wt. The total amount of copper (in μg) in skin is given in parentheses below its copper concentration. Abbreviation used: ND, not determined.

Tissue	3				54			
	Untreated $+/Y$	Treated $+/Y$	Untreated Mo^{blo}/Y	Treated Mo^{blo}/Y	Untreated $+/Y$	Treated $+/Y$	Untreated Mo^{blo}/Y	Treated Mo^{blo}/Y
Brain	7.5	10.5	2.4	4.7	13.4	16.1	4.3	7.0
Liver	113.7	540.9	9.4	113.5	15.0	19.3	15.8	14.8
Kidney	11.6	18.9	25.6	99.0	14.9	17.1	53.2	57.9
Spleen	9.6	22.2	3.6	10.8	4.6	4.2	2.2	2.1
Lung	6.3	10.6	4.9	12.8	7.5	5.1	9.0	9.9
Heart	19.2	23.2	11.4	21.9	27.2	25.7	19.0	16.7
Skin	4.1 (2.3)	14.6 (9.2)	3.0 (1.1)	23.9 (12.8)	ND	ND	ND	ND
Muscle	4.3	5.4	2.6	5.5	3.2	2.4	2.0	2.3
Testis	6.8	6.8	5.6	23.3	ND	ND	ND	ND
Caeruloplasmin	0.447	0.434	0.165	0.420	0.472	0.348	0.290	0.267

Fig. 1. *Weight gain of blotchy mice after copper therapy*

Mice were injected subcutaneously with $50\mu\text{g}$ of copper at 7 days of age. The copper solution used, the number of observations (in parentheses), and the growth curves are as follows. Upper group: +/Y, no treatment (10) —; $\text{Cu}^+\text{Seb(p)}$ (7) ----; $\text{Cu}^{2+}\text{Seb(p)}$ (6) ····. Lower group: $\text{Mo}^{\text{blo}}/\text{Y}$, no treatment (10) —; $\text{Cu}^+\text{Seb(p)}$ (8) ----; $\text{Cu}^{2+}\text{Seb(p)}$ (6) ····. The s.d. values for +/Y untreated mice are shown by the vertical bars. Student's *t* test was used to assess significance. All combinations of +/Y growth curves: $P > 0.10$ from day 7 to day 61. All combinations of $\text{Mo}^{\text{blo}}/\text{Y}$ growth curves: $P > 0.40$ from day 7 to day 61. All +/Y mice combined versus all $\text{Mo}^{\text{blo}}/\text{Y}$ mice combined: $P < 0.05$ at day 7, $P > 0.05$ from day 10 to day 61 inclusive.

Table 4. *Distribution of ^{64}Cu in 9–12-day-old blotchy mice*

Two +/Y, two $\text{Mo}^{\text{blo}}/\text{Y}$ and two $\text{Mo}^{\text{blo}}/+$ mice for each time interval received in intracardiac injection of $0.19\text{--}0.41\mu\text{Ci}$ of ^{64}Cu ($0.43\mu\text{g}$ of total copper as $^{64}\text{CuCl}_2$) in 0.9% NaCl and were killed at 10 min, 5, $10\frac{1}{2}$ and 21 h after injection. Mice to be killed at 48 h were placed back with the mother after injection. The means of two observations are expressed as a percentage of the recovered dose for mice killed at 10 min–21 h, and as a percentage of the administered dose for mice killed at 48 h, as urine radioactivity could not be measured. Results for blood are expressed as a percentage of the recovered dose/g. For all the mice used, the average total recovery of the administered dose was $102 \pm 3.65\%$. Faeces never contained measurable radioactivity.

Time after injection	Tissue ...	Blood	Urine	Gut	Brain	Liver	Kidney	Spleen	Lungs	Heart	Skin	Remaining carcass
Mouse												
10 min	+/Y	20.93	0.17	7.87	0.41	52.14	2.98	0.49	2.28	0.63	7.22	22.30
	$\text{Mo}^{\text{blo}}/\text{Y}$	38.35	0.13	4.97	0.64	46.20	2.24	0.48	2.44	1.29	8.94	28.18
	$\text{Mo}^{\text{blo}}/+$	44.99	0.24	7.70	0.46	41.87	3.13	0.28	2.53	1.07	8.20	28.70
5 h	+/Y	8.96	0.41	12.83	0.64	49.33	1.71	0.66	0.98	0.58	11.04	20.74
	$\text{Mo}^{\text{blo}}/\text{Y}$	9.66	0.45	11.83	0.89	36.87	4.95	0.54	1.25	0.85	12.23	29.26
	$\text{Mo}^{\text{blo}}/+$	9.53	0.68	19.61	1.01	23.88	5.25	0.86	1.15	0.87	14.09	31.23
$10\frac{1}{2}$ h	+/Y	5.53	0.54	11.78	0.70	59.24	1.47	0.63	0.72	0.40	8.08	15.77
	$\text{Mo}^{\text{blo}}/\text{Y}$	8.73	0.54	11.83	0.73	38.48	4.87	0.50	1.39	0.69	11.97	28.32
	$\text{Mo}^{\text{blo}}/+$	11.08	0.50	17.59	0.78	26.84	4.23	0.53	1.83	0.82	13.77	31.80
21 h	+/Y	4.39	0.91	11.68	0.87	61.87	1.27	0.53	0.70	0.38	6.87	14.32
	$\text{Mo}^{\text{blo}}/\text{Y}$	6.04	0.53	25.22	1.03	12.81	5.62	0.36	2.07	1.52	16.71	32.91
	$\text{Mo}^{\text{blo}}/+$	8.13	0.94	19.18	1.04	19.09	6.44	0.64	1.15	0.73	17.88	32.34
48 h	+/Y	7.23	—	9.25	1.77	45.12	1.13	0.42	0.89	0.67	15.88	23.11
	$\text{Mo}^{\text{blo}}/\text{Y}$	4.09	—	19.07	1.49	8.02	5.72	0.24	1.39	0.78	21.24	40.40
	$\text{Mo}^{\text{blo}}/+$	8.29	—	15.81	1.83	8.15	6.43	0.33	1.19	0.92	24.19	39.72

Fig. 2. Distribution of ^{64}Cu in 9–12-day-old blotchy mice. Two $+/Y$ (■), two Mo^{blo}/Y (●) and two $Mo^{blo}/+$ (▲) mice for each time interval received an intracardiac injection of $0.19\text{--}0.41\mu\text{Ci}$ of ^{64}Cu ($0.43\mu\text{g}$ of total copper as CuCl_2) in 0.9% NaCl and were killed at 10 min, 5, 10½ and 21 h after injection. Mice to be killed at 48 h were placed back with the mother after injection, and the results for these mice are expressed as a percentage of the administered dose, as excretion prevented a total recovery of the dose. (a) Kidney; (b) skin; (c) brain; (d) liver.

later. The distribution in individual organs of Mo^{blo}/Y and $Mo^{blo}/+$ mice compared with $+/Y$ mice showed three different patterns: (1) kidney (Fig. 2a), gut, lung, heart, skin (Fig. 2b) and carcass accumulated ^{64}Cu excessively; (2) brain (Fig. 2c) and spleen showed no significant differences; (3) liver (Fig. 2d) lost radioisotope rapidly after apparently normal initial uptake.

Low amounts of ^{64}Cu recovered in the ligated stomach and duodenum of Mo^{blo}/Y and $Mo^{blo}/+$ mice at 21 h after intracardiac injection demonstrated that the loss of ^{64}Cu from the liver was not due to biliary excretion. [Percentage of the recovered dose in liver: $+/Y$, 51.8 (2); Mo^{blo}/Y , 21.4 (2); $Mo^{blo}/+$, 13.9 (1); stomach and duodenum: $+/Y$, 15.5 (2); Mo^{blo}/Y , 7.7 (2); $Mo^{blo}/+$, 10.1 (1). The number of observations is in parentheses.]

Discussion

These experiments demonstrate a marked alteration of copper transport and distribution in Mo^{blo}/Y and $Mo^{blo}/+$ mice. In almost every case, the nature of this alteration is very similar to that occurring in Mo^{br}/Y and $Mo^{br}/+$ mice of the same age (Camakaris *et al.*, 1979; Mann *et al.*, 1979a,b). This finding is compatible with allelism of the Mo^{blo} and Mo^{br} mutations and continues to suggest homology with the mutation in Menkes' syndrome. Ultimate proof of allelism and of homology could be achieved by cell-hybridization experiments in which both X-chromosomes are active in the same cell. Alternatively, demonstration that the basic molecular defect involves the same protein in all three mutations would achieve the same objective.

The differences that are present between the results in blotchy and brindled mice are largely ones of the degree of alteration, and a comparison of the various assays is shown in Table 5. This shows that, in the majority of cases, Mo^{blo}/Y mice are less abnormal than Mo^{br}/Y mice, as would be expected from their greater viability and less severe overt pathology. In comparing the assays in $Mo^{blo}/+$ mice and $Mo^{br}/+$ mice, no particular trend is present (Table 5).

Although the subcutaneous injection of 7-day-old Mo^{blo}/Y mice with $50\mu\text{g}$ of copper ameliorated many of the symptoms of copper deficiency, as it did in Mo^{br}/Y mice (Mann *et al.*, 1979a), it had no effect on the rate of weight gain. This was unexpected, as the same copper therapy improved the rate of weight gain in the more severely affected Mo^{br}/Y mice, and this was to an even greater extent than that seen in Mo^{blo}/Y mice (Mann *et al.*, 1979a). The success of the treatment in Mo^{br}/Y mice was attributed to the abnormally high retention of copper at the site of injection acting as a depot dose, allowing a slow release of copper to the tissues (Mann *et al.*, 1979a). This depot effect may be less marked in Mo^{blo}/Y mice.

In previous studies, the distribution of ^{64}Cu over 48 h in many organs of Mo^{br}/Y and $Mo^{br}/+$ mice indicated that the initial uptake of copper into the organs was normal, and that the later mechanisms of copper distribution were at fault (Mann *et al.*, 1979b). This situation is again apparent in blotchy mice, and

Table 5. *Comparison of assays of 9–11-day-old blotchy mice with those of brindled mice*

The values presented below are the percentages of the values for +/Y mice in Table 1, referring to copper concentrations, haemoglobin concentration and caeruloplasmin oxidase activity. Data for brindled mice are taken from Camakaris *et al.* (1979), except those of ileal epithelial cells and whole gut (plus luminal contents).

	Mo^{blo}/Y	Mo^{br}/Y	$Mo^{blo}/+$	$Mo^{br}/+$
Whole gut	529	575	359	375
Ileal luminal contents	1503	1583	—	—
Ileal epithelial cells	998	861	—	—
Brain	32	27	53	63
Liver	8	8	12	15
Kidney	221	318	336	721
Spleen	38	25	51	68
Muscle	60	49	84	103
Heart	59	29	82	64
Testis	82	125	—	—
Skin	73	63	98	129
Plasma	53	32	74	76
Erythrocytes	45	39	60	51
Urine	107	90	211	149
Haemoglobin	104	92	99	92
Caeruloplasmin	37	19	87	75

can be more clearly seen in the following comparison. The distribution of ^{64}Cu among organs of +/Y mice was similar to that of pre-existing copper from 10 min through to 48 h. In Mo^{blo}/Y and $Mo^{blo}/+$ mice, the distribution of ^{64}Cu at 10 min conformed to the pattern in +/Y mice. However, its distribution altered with time to become similar to the abnormal distribution of pre-existing copper by 48 h [Figs. 2a–2d; Table 4 and Table 1 (i)(b)]. Those organs of Mo^{blo}/Y and $Mo^{blo}/+$ mice that accumulated excessive amounts of copper over this time parallel the behaviour of cultured cells derived from Mo^{br}/Y mice and humans with Menkes' syndrome (Camakaris *et al.*, 1980). Collectively, these observations indicate that the excessive accumulation of copper is a rather fundamental disturbance caused by the mouse and human mutations.

The simplest hypothesis to explain the majority of the findings in mottled mouse mutants and in Menkes' syndrome would be a mutation primarily affecting an intracellular copper-binding molecule (such as metallothionein), causing excessively avid binding or excessive production of the binding protein (Danks, 1977). This hypothesis is probably too simple, and the excessive accumulation of copper may be related to superinduction of metallothionein production secondary to some other disturbance in the normal processes of intracellular copper transport. If this were true, then the secondary status of the phenomenon of copper accumulation within cells would make its rather varied behaviour in different organs, e.g. liver, and the degree of its

behaviour in heterozygous females, somewhat less surprising.

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