Copper Metabolism in Mottled Mouse Mutants

COPPER CONCENTRATIONS IN TISSUES DURING DEVELOPMENT

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The copper content of various organs of 'brindled' female heterozygotes and male mice affected by this X-linked mutation are documented at the last day of intrauterine development, at 1 day after birth and at 11 days of age. The findings indicate defective placental transfer of copper *in utero*, and an even more marked defect in intestinal absorption of copper after birth. In addition there is an abnormal distribution of copper among the tissues of the body once it is absorbed. The mutation produces abnormal accumulation of copper in kidney, in gut mucosa and in testis, whereas liver, brain, plasma and most other organs show diminished copper concentrations. The intestinal malabsorption of copper is accompanied by accumulation of abnormal amounts of the metal in the intestinal-mucosa cells. Copper concentrations in both mucosa and luminal contents rise progressively from duodenum to ileum. Defective upper-intestinal absorption, consequent progressive increase in luminal copper concentration and pinocytosis in the ileum would seem to explain the findings. Radioisotopic studies eliminated the possibility of excessive excretion of copper in bile or across the intestinal mucosa. Detailed comparison with findings in humans with Menkes' syndrome is difficult because of the different stages of development at which the studies have been performed, but the results seem in general to conform very satisfactorily. Those differences seen are probably explicable by known species differences. All the findings are in accord with a hypothesis that the basic defect involves accumulation and retention of copper in the cells of affected tissues such as kidney, gut mucosa and placenta.

Mottled mice, especially the variant brindled, have been shown by Hunt (1974, 1976) to provide an animal model of Menkes' syndrome in humans, with similarities so great as to suggest genetic homology of the X-linked mutations in mouse and man.

Danks *et al.* (1972) demonstrated decreased absorption of ⁶⁴Cu in Menkes' syndrome, and the finding of high concentrations of copper in duodenal-mucosal biopsies (Danks *et al.*, 1973) led to postulation of defective transport within the mucosal cell or defective release into the bloodstream after normal uptake from the lumen. Dekaban *et al.* (1975) have measured the degree of copper malabsorption more accurately and have shown about 20% of normal absorptive capacity. Failure of parenteral copper-replacement therapy to cure the disease and the existence of mosaic skin changes in humans (Volpintesta, 1974) and mice indicate that the disease is more complex than a simple copper malabsorption, with involvement of multiple cell types with the basic defect (Danks, 1977). Hunt

(1974) has described a similar defect in intestinal copper transport in brindled mice.

The aim of the present study is to fill in some of the many gaps in our understanding of copper metabolism in the pre- and post-natal brindled mouse mutant and to compare the findings with those in Menkes' syndrome in order to gain a greater insight into the basic defect in these mice and in humans with Menkes' syndrome.

Experimental

Mice used were the brindled variants of the Mottled series of sex-linked alleles. Affected males die at 14 days *post partum*. Original mating pairs of these mice were kindly sent to us by Dr. David Hunt, Queen Mary College, University of London, London, U.K. Adult mice were 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. The mice were housed in plastic boxes with steel tops.

All copper determinations were made with the Varian-Techtron CRA-90 atomic absorption spectrophotometer, containing the BC-6 automatic background-subtract module.

All glassware was acid-washed and surgical instruments were washed in EDTA as described by Stevens (1972).

Copper in tissues and gut contents

Whole tissues and gut contents were processed and measured by the methods described by Stevens (1972). Results are expressed as μg of copper/g dry wt. Tissue samples were weighed with a Mettler M5 micro-analytical balance. BDH 'Aristar' HNO_3 was used in all tissue and cell digestions.

Copper in blood

Blood was collected from individual mice in 0.5 ml heparinized acid-washed Beckman microfuge tubes, and was centrifuged at 9000g/4 min in a Beckman microfuge. Portions (25 μl) of plasma were removed, digested in 100 μl of concentrated HNO_3 and copper was measured. The remaining plasma was removed, the volume of the erythrocyte pellet measured, and the pellet was digested in 400 μl of concentrated HNO_3 . Serum of foetal and 1-day-old mice was measured directly because only small quantities could be collected. Results are expressed as μg of copper/100 ml.

Copper in gut-mucosal cells

Duodenum was taken from the pyloric sphincter to the beginning of the upper jejunal loop, and the remaining small intestine was divided into three sections of approximately equal length. These segments were removed, ligated and injected with a solution containing 0.625% (w/v) hyaluronidase (Sigma Chemical Co., St. Louis, MO, U.S.A.; bovine testis type 1), and 0.025% (w/v) collagenase (Sigma; *Clostridium histolyticum* type 1) in 0.9% NaCl, and then incubated under CO_2 in a humidified incubator at 37°C for 40 min in phosphate-buffered saline (Dulbecco & Vogt, 1954). Cells were harvested by centrifugation at 1000g/5 min, washed in phosphate-buffered saline and then 0.9% NaCl, and a cell count was made by using a Hawksley Haemocytometer. After centrifugation at 1000g/5 min and removal of the supernatant, the cell pellet was digested in HNO_3 and copper was measured. Results are expressed as μg of copper/ 10^9 cells. Multiplication of this by a factor of 1.82 converts it into $\mu\text{g/g}$ dry wt.

Urine

Urine was collected from individual mice by bladder puncture. Creatinine was determined by an

ultra-micro adaptation of the Jaffé reaction (Edwards & Whyte, 1959). Urinary copper was measured directly. Results are expressed as μg of copper/mmol of creatinine.

Haemoglobin

Haemoglobin was determined for individual mice by using the cyanmethaemoglobin method with the commercial converting reagent Zapoglobin (Coulter Electronics, Harpenden, Herts., U.K.). Results are expressed as g/100 ml of blood.

Assay of caeruloplasmin oxidase activity

The method was based on that of Bingley & Dick (1969). The optimal pH for mouse caeruloplasmin oxidase activity was determined to be pH 5.25 (J. R. Mann, unpublished work). The assay was scaled down so that 50 μl of mouse serum could be assayed. Results are expressed as A_{570} units. As pure mouse caeruloplasmin has not been characterized the conventional conversion into mg/100 ml has not been made.

Intragastric injections

Mice were anaesthetized with diethyl ether. The abdomen was opened by a mid-line incision and solutions were injected into the stomach with a micro-syringe. The abdomen was resealed by stitching and 5 h later the mice were killed and their small intestine was removed.

(i) $^{51}\text{CrCl}_2$. Either 10 μl of this, containing 0.1 μCi (11-day-old mice), or 100 μl containing 1 μCi (60-day-old mice), was injected. The specific radioactivity was 100 $\mu\text{Ci}/\mu\text{g}$ of Cr (The Radiochemical Centre, Amersham, Bucks., U.K.). The small intestine was cut into three equal-length segments. Two normal and two mutant 11-day-old mice and three normal adult mice were injected. Each segment was washed with non-radioactive 0.02M- $\text{CrK}(\text{SO}_4)_2$ and the sections were counted directly for radioactivity in an LKB Ultrogamma 1280 counter. Results were expressed as a percentage of the administered dose.

(ii) CuCl_2 . Two normal 11-day-old mice were injected with 100 μl containing 15 μg of copper. The CuCl_2 was in a solution of 50% (v/v) cow's milk. Mucosal cells were isolated from the duodenum, jejunum and ileum as described above.

Intracardiac injection of ^{64}Cu

$^{64}\text{CuCl}_2$ was purchased as a sterile solution in 0.9% NaCl from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. Before the experiment 11-day-old mutant and normal mice were starved overnight, and under ether anaesthesia the abdomen was opened by a mid-line incision and the intestine ligated so as to separate the duodenum and stomach from the rest of the alimentary tract. Then 6 μl containing approx. 4 μCi

Table 1. *Copper and haemoglobin concentrations and caeruloplasmin oxidase activity in 11-day-old mice*

Normal, mutant and heterozygote mice (11 days old) were killed and whole organs removed, and copper was determined as described in the Experimental section. Copper concentration is expressed as $\mu\text{g/g}$ dry wt. of tissue, or $\mu\text{g}/100\text{ml}$ for plasma and erythrocytes or $\mu\text{g}/\text{mmol}$ of creatinine for urine. Haemoglobin is expressed as $\text{g}/100\text{ml}$ and caeruloplasmin oxidase activity as A_{570} units. Results are means $\pm$ S.D. with the numbers of observations in parentheses. Caeruloplasmin oxidase activity was determined on the pooled sera of four mice in each group. Student's *t* test was used to assess significance.

	Normal Mo ⁺	Mutant Mo ^{br/y}	Heterozygote Mo ^{br/+}	<i>P</i> values : + versus br/y; + versus br/+; br/y versus br/+
Brain	12.0 $\pm$ 2.5 (6)	3.2 $\pm$ 0.4 (6)	7.5 $\pm$ 0.6 (6)	<0.01 <0.01 <0.01
Liver	169.2 $\pm$ 92.5 (6)	13.3 $\pm$ 1.1 (5)	24.6 $\pm$ 2.9 (5)	<0.01 <0.01 <0.01
Kidney	15.6 $\pm$ 1.3 (6)	49.6 $\pm$ 9.5 (6)	112.4 $\pm$ 28.1 (6)	<0.01 <0.01 <0.01
Spleen	16.5 $\pm$ 4.0 (5)	4.1 $\pm$ 0.6 (6)	11.3 $\pm$ 2.8 (6)	<0.01 <0.05 <0.01
Muscle	8.7 $\pm$ 1.2 (6)	4.3 $\pm$ 1.0 (5)	9.0 $\pm$ 1.7 (6)	<0.01 >0.05 <0.01
Heart	23.1 $\pm$ 3.4 (4)	6.8 $\pm$ 1.0 (4)	14.8 $\pm$ 3.6 (4)	<0.01 <0.05 <0.01
Testis	10.9 $\pm$ 0.8 (4)	13.6 $\pm$ 0.9 (4)	—	<0.01 <0.01
Skin	4.1 $\pm$ 0.4 (4)	2.6 $\pm$ 1.1 (4)	5.3 $\pm$ 1.5 (4)	<0.05 >0.05 <0.01
Stomach	18.9 $\pm$ 3.0 (3)	13.0 $\pm$ 0.9 (4)	25.0 $\pm$ 3.0 (4)	<0.05 <0.05 <0.01
Plasma	76.0 $\pm$ 5.0 (4)	24.0 $\pm$ 5.0 (4)	58.0 $\pm$ 4.0 (5)	<0.01 <0.01 <0.01
Erythrocytes	200.0 $\pm$ 28.0 (4)	77.0 $\pm$ 12.0 (4)	102.0 $\pm$ 11.0 (5)	<0.01 <0.01 <0.05
Urine	66.4 $\pm$ 10.2 (7)	59.5 $\pm$ 16.1 (5)	99.0 $\pm$ 24.7 (5)	>0.05 <0.01 <0.05
Haemoglobin	11.9 $\pm$ 2.0 (6)	10.9 $\pm$ 0.7 (6)	10.9 $\pm$ 0.9 (6)	>0.05 >0.05 >0.05
Caeruloplasmin	0.36	0.07	0.27	

of ^{64}Cu was injected into the blood of each mouse by intracardiac injection with a micro-syringe. The abdomen was resealed by stitching and 21 h later the mice were killed. The sections of gut plus contents above and below the ligation were counted separately for radioactivity in an LKB Ultrogamma 1280 counter by using programmed decay correction (by using a half-life of ^{64}Cu of 12.8 h) and automatic background subtraction. Results were expressed as a percentage of the administered dose.

Foetal and neonatal mice

Foetuses were taken during the last 2 days of pregnancy, i.e. 20–21 days gestation. Although the foetuses could be easily sexed, they could not be identified accurately as mutants by the presence of curly whiskers (Fraser *et al.*, 1953; Phillips, 1961). The copper distribution in different organs of male foetuses was found to fall into discrete classes and could be used for genotypic classification. The validity of this classification has been verified by

tissue culture. Cultured cells from 10-day-old brindled mice have been shown to accumulate high concentrations of copper (Danks, 1977). Cultured foetal cells derived from foetuses classified as mutants possessed copper concentrations of $0.30 \mu\text{g}/10^6$ cells, compared with $0.05 \mu\text{g}/10^6$ cells in foetuses classified as normal, thus confirming the classification. Among female foetuses and 1-day-old pups, a clear-cut segregation into two classes was observed in relation to liver copper content, and it was assumed that the group with the low liver copper content were heterozygotes. Affected males at 1 day of age could be easily identified by the presence of curly whiskers.

Results

Copper concentrations shown in Tables 1 and 2 demonstrate a severe state of copper deficiency in many tissues of mutant mice, with the exception of kidney and the distal part of the small intestine,

Table 2. *Copper concentrations in 11-day-old mice: small-intestinal epithelial cells*

Small-intestinal-epithelial cells from various segments were isolated and copper was determined as described in the Experimental section. Results are means $\pm$ S.D., with the numbers of observations in parentheses.

	Copper content ($\mu\text{g}/10^9$ cells)		
	Normal Mo ⁺	Mutant Mo ^{br/y}	Heterozygote Mo ^{br/+}
Duodenum	8.5 ± 1.5 (5)	11.5 ± 3.1 (6)	14.7 ± 3.7 (5)
Jejunum	7.9 ± 2.9 (2)	59.3 ± 54.0 (2)	13.0 ± 0.7 (2)
Jejunum-ileum	14.9 ± 0.3 (2)	141.1 ± 34.1 (2)	41.5 ± 41.3 (2)
Ileum	9.7 ± 0.7 (2)	199.4 ± 2.9 (2)	51.6 ± 36.9 (2)

where copper concentrations are elevated well above normal, and testis, where copper concentrations are slightly elevated. Caeruloplasmin oxidase activity was found to be more decreased in the mutants than previously reported (Hunt, 1974).

Urinary copper is as high in mutants as in normals, even though plasma copper is one-third of normal. Heterozygote females show even higher concentrations than mutants in both kidney and urine (Table 1).

Our initial attempts to measure copper in the intestine were restricted to the duodenum, where this whole segment was washed, contents were removed, and copper contents measured as $\mu\text{g}/\text{g}$ dry wt. of tissue. This method, however, yielded highly variable results, and it was apparent that a more satisfactory method of measuring intestinal copper was required. The advantages of measuring copper in isolated cells are (a) specificity in that absorptive cells and not the connective tissue and muscle layers of the gut wall are included in the measurements, and (b) the avoidance of any gross contamination from the contents of the lumen (Table 3). The avoidance of contamination from lumen contents was demonstrated in an experiment where adult normal mice were fed for 4 days on a high-copper diet containing $650 \mu\text{g}/\text{g}$ dry wt., and small-intestinal epithelial cells were isolated and measured as above. The amounts of copper in the cells thus treated were only slightly higher than those from normally fed mice, receiving a diet containing $17 \mu\text{g}$ of copper/g dry wt. [high-copper diet 12.1 ± 3.9 (6) μg of copper/ 10^9 cells; low-copper diet 9.8 ± 3.6 (6) μg of copper/ 10^9 cells: values are means $\pm$ S.D., numbers of observations in parentheses].

The concentration of copper in small-intestine

Table 3. *Copper concentrations in 11-day-old mice: gastrointestinal-lumen contents*

Copper in lumen contents was measured as described in the Experimental section. Results are means $\pm$ S.D., with the numbers of observations in parentheses, followed by the range of values obtained.

	Copper content ($\mu\text{g}/\text{g}$ dry wt.)		
	Normal Mo ⁺	Mutant Mo ^{br/y}	Heterozygote Mo ^{br/+}
Stomach	4.9 ± 1.1 (4) 3.3 – 5.8	7.4 ± 2.0 (4) 5.1 – 9.3	6.1 ± 2.7 (4) 2.7 – 7.8
Duodenum	16.4 ± 2.8 (7) 12.7 – 19.7	40.6 ± 9.8 (8) 32.7 – 63.1	44.2 ± 16.1 (7) 23.1 – 68.6
Jejunum	23.0 ± 14.7 (5) 9.7 – 48.1	241.6 ± 327.8 (6) 40.9 – 876.9	56.6 ± 8.8 (4) 46.9 – 66.0
Jejunum-ileum	41.8 ± 14.4 (5) 18.2 – 56.9	299.2 ± 283.1 (6) 58.1 – 843.7	175.8 ± 41.1 (4) 122.1 – 214.7
Ileum	44.4 ± 10.8 (5) 33.1 – 59.6	702.9 ± 381.1 (10) 384.5 – 1504.4	167.8 ± 53.9 (3) 120.1 – 226.3
Caecum	Not tested	116.7 ± 70.7 (2)	Not tested
Colon-rectum	52.1 ± 13.3 (5) 29.8 – 64.3	209.2 ± 114.3 (5) 56.5 – 334.3	85.7 ± 15.3 (4) 71.8 – 107.5

lumen contents from mutant mice increases progressively from stomach to caecum (Table 3), and this situation also occurs for the epithelial cells (Table 2). Sources of lumen-content copper could be biliary, via excretion through the gut wall, or non-absorbed copper. To test whether luminal copper represents copper lost via excretion in the bile or through the gut wall, the small intestine was ligated below the duodenum and ^{64}Cu was injected into the blood as described in the Experimental section.

After 21 h, 16.4% of the administered dose was recovered above the ligation in the normal mouse and 5.2% was recovered above the ligation in the mutant mouse. Assuming that radioactivity recovered above the intestinal ligation in ^{64}Cu -injected mice represents the total copper excreted in bile, these data indicate that no abnormally large loss of copper occurred via this route in mutants. Furthermore, measurements on pooled gall bladders from 11-day-

old mice show that there is a low amount of copper in this tissue of the mutant mouse [normal $46.3 \mu\text{g/g}$ dry wt. (5); brindled $12.5 \mu\text{g/g}$ dry wt. (5)].

Radioactivity in the gut below the ligation was 2.2% of the administered dose in the normal mouse and 6.2% of the administered dose in the mutant mouse. This indicates that excretion via the gut wall is insignificant with respect to contributing copper to the gut lumen of mutants. It therefore appears likely that the high lumen content of copper is primarily due to malabsorption.

Table 2 shows that mutant mice accumulate very high amounts of copper in epithelial cells derived from distal small intestine, particularly ileum. Clark (1959) has reported evidence for the existence of pinocytosis of protein and colloidal materials in the distal region of the small intestine of suckling rats and mice, whereas Mistilis & Mearrick (1969) suggested that pinocytosis was responsible for the ileal absorption of copper in suckling rats. It seemed

Table 4. *Copper concentrations of foetal and 1-day-old mice*

Foetuses at 20–21 days gestation and 1-day-old newborns were killed and copper concentration measured as described in the Experimental section. Copper concentration is expressed as $\mu\text{g/g}$ dry wt. of tissue, or $\mu\text{g}/100\text{ml}$ for plasma. Results are means $\pm$ S.D., with the numbers of observations in parentheses. Student's *t* test was used to assess significance.

	Normal Mo ⁺	Mutant Mo ^{br/y}	Heterozygote Mo ^{br/+}	<i>P</i> values: + versus br/y; + versus br/+; br/y versus br/+
Foetus				
Brain	4.6 $\pm$ 0.3 (3)	2.6 $\pm$ 0.4 (3)	Not tested	<0.01
Liver	63.5 $\pm$ 13.2 (11)	27.2 $\pm$ 10.2 (7)	26.6 $\pm$ 9.5 (6)	<0.01 <0.01 >0.05
Kidney	7.2 $\pm$ 1.3 (3)	11.2 $\pm$ 2.2 (3)	Not tested	<0.05
Proximal small-intestine mucosa	29.4 $\pm$ 13.0 (8)	22.7 $\pm$ 4.1 (4)	19.6 $\pm$ 1.8 (6)	>0.05 >0.05 >0.05
Distal small-intestine mucosa	28.1 $\pm$ 7.0 (8)	17.6 $\pm$ 5.8 (4)	17.9 $\pm$ 5.9 (6)	<0.05 <0.05 >0.05
Placenta	11.0 $\pm$ 2.4 (8)	22.3 $\pm$ 5.1 (4)	13.4 $\pm$ 3.3 (6)	<0.01 >0.05 <0.01
Serum	35.5 $\pm$ 5.5 (5)	16.7 $\pm$ 3.1 (5)	Not tested	<0.01
1 day old				
Brain	9.7 $\pm$ 2.0 (9)	5.3 $\pm$ 0.6 (4)	5.9 $\pm$ 0.3 (5)	<0.01 <0.01 >0.05
Liver	118.8 $\pm$ 70.2 (9)	37.9 $\pm$ 9.2 (4)	40.4 $\pm$ 20.3 (5)	<0.05 <0.05 >0.05
Kidney	24.4 $\pm$ 5.3 (9)	45.5 $\pm$ 4.6 (4)	64.5 $\pm$ 23.5 (5)	<0.01 <0.01 >0.05
Proximal small-intestine mucosa	24.7 $\pm$ 4.8 (3)	41.3 $\pm$ 5.6 (3)	Not tested	<0.01
Distal small-intestine mucosa	26.0 $\pm$ 10.2 (3)	29.7 $\pm$ 6.8 (3)	Not tested	>0.05
Serum	70.0 $\pm$ 13.0 (3)	21.0 $\pm$ 2.5 (3)	Not tested	<0.01

Table 5. Comparison of copper concentrations in normal and mutant mice at different stages of development

This Table summarizes data from Tables 1 and 4 and shows mean copper concentrations in $\mu\text{g/g}$ dry wt. of tissue or $\mu\text{g}/100\text{ml}$ of serum.

	Brain	Liver	Kidney	Serum
Normal (foetus)	4.6	63.5	7.2	35.5
Normal (1 day)	9.7	118.8	24.4	70.0
Normal (11 days)	12.0	169.2	15.6	76.0
Mutant (foetus)	2.6	27.2	11.2	16.7
Mutant (1 day)	5.3	37.9	45.5	21.0
Mutant (11 days)	3.2	13.3	49.6	24.0

likely that the high amounts of copper in ileal epithelial cells from mutants could be a result of pinocytosis. To test this idea the non-absorbable radioisotope ^{51}Cr (Visek *et al.*, 1953) was injected into the stomach of 11-day-old brindled and normal mice and into the stomach of adult mice. A significant percentage of the administered dose (20–25%) was taken up into the mucosa of distal small intestine of the young mice, but very little (1–2%) was taken up by adult mice. In neither case was there any significant systemic absorption of ^{51}Cr .

In another experiment large doses of copper were injected into the stomach of 11-day-old normal mice, and it was shown that 5 h later epithelial cells derived from the ileum contained $33.6\mu\text{g}$ of copper/ 10^9 cells, compared with $6.6\mu\text{g}$ of copper/ 10^9 duodenal epithelial cells. These experiments support the idea that pinocytosis is a significant factor in the great accumulation of copper in the distal region of the small intestine of young mutant mice.

Foetal copper concentrations were measured in order to assess copper status when not dependent on gut absorption. Table 4 shows that, with the exception of the kidney, copper concentrations in the foetus are low, whereas placenta concentrations are elevated, suggesting a placental transport defect.

Comparison of results at 11 days *post partum* with those at 1 day, in mutant mice, showed that liver and brain copper concentrations actually decreased, whereas serum copper concentrations remained relatively constant (Table 5), and normal mice showed increases in liver and brain copper. Copper concentrations in proximal and distal small intestine of 1-day-old mutant pups are normal (Table 4). After a 4-fold increase at birth the copper concentrations in the mutant kidney do not significantly change by 11 days (Table 5).

Discussion

The mottled series of mouse mutants are important because they provide a homologue to human Menkes' syndrome, but are even more important because of their potential as a means of understanding normal intracellular copper metabolism.

The findings in those tissues that were studied at 20–21 days gestation, at 1 day of age and at 11 days of age fall into two distinct groups. Copper concentrations in brain and liver rise progressively throughout the three stages studied in normal mice, but in the mutants the copper concentrations of these organs are lower than normal in the foetus, rise a little by 1 day of age and then fall again by 11 days of age. In contrast, the copper concentration of kidney and of gut mucosa in normal mice is lower at 11 days of age than at 1 day of age, whereas in the mutants it is higher at 11 days for gut and unaltered for kidney. Other tissues which have been studied at 11 days of age only all fit into the pattern of low copper content in the mutant, with the exception of testis, which contains slightly elevated copper concentrations.

The fact that the placenta has an increased copper content and that the gut mucosa shows a marked increase in copper content after oral feeding commences, along with the observation of diminished intestinal absorption of copper (Hunt, 1974; Camakaris *et al.*, 1978; Evans & Reis, 1978) suggests that the brindled mutant is deprived of copper both *in utero* and postnatally because of deficient function of the respective transport organ. However, the whole pattern described cannot be attributed merely to defective absorption of copper and must be seen as a compounding of defective absorption and abnormal distribution of copper that is absorbed.

The progressive rise in copper concentration in the luminal content of the intestine (Table 3) is probably due to defective absorption specific to copper. Excessive biliary intestinal excretion has been eliminated by radioisotopic studies. The progressively increasing copper concentration is also observed in small-intestinal-mucosal cells (Table 2) and is likely to be a consequence of a mass-action effect exerted by the increasing copper concentration present in the lumen. Non-specific absorption of copper in suckling mice by pinocytosis in the distal part of the small intestine would also significantly contribute to high concentrations in the mucosa of this region. Evidence for pinocytosis in distal small intestine is shown by the ^{51}Cr accumula-

tion in both normal and mutant 11-day-old mice and the high copper contents of mucosal cells observed in normal suckling mice fed with high concentrations of copper. Significant absorption of copper by the ileum is indicated by the sharp fall in lumen copper concentration in the caecum and colon (Table 3). Differences in turnover time in different regions of the small intestine could result in differences in observed copper concentration. However, in the rat, cell turnover time has been shown to be approximately the same in proximal and distal small intestine (Leblond & Stevens, 1948).

The very high copper concentration in mutant kidneys presumably reflects retention of filtered copper in renal-tubule cells comparable with what is observed in the small intestine. Evans & Wiedersanders (1967) reported that suckling rats have a high proportion of their serum copper not bound to caeruloplasmin. It is probable that this situation also occurs in suckling mice, and in this form copper would be readily filtered into the renal tubules and a defective reabsorption of this copper would result in accumulation in renal-tubule lumen and tubular cells. Defective reabsorption would explain why urinary excretion of copper in the mutants is the same as in normals even though the copper concentration in plasma of mutants is one-third of normal.

The findings in the heterozygous females are really very remarkable. The particularly high concentration of copper seen in the kidneys may be indicating the extent of copper retention of which mutant cells are capable given an adequate supply from the bloodstream. In the gut one must presume that the 50% of the cells that are normal in the heterozygote will allow substantial absorption of copper. This means that renal filtration will be increased compared with that in the mutant males and allow the 50% population of mutant cells in renal tubules to express their maximum capacity for retaining copper.

Comparisons with human Menkes' syndrome are difficult because tissues from affected human patients become available at stages of development different from those studied in the mice. Aborted human foetuses have been studied at mid-gestation and patients have been studied at autopsy after death at ages varying from 6 months to 3 years. Species differences add further complexity. The mouse is known to be born with low liver copper concentrations, which rise very rapidly after birth (Prins & Van den Hamer, 1978), and this trend is also seen in the data of the present study. However, the human is born with very high concentrations of copper (Widdowson *et al.*, 1972), which fall progressively over the first few months of life (Brückmann & Zondek, 1939). In addition the phenomenon of pinocytosis in distal small intestine of suckling rodents undoubtedly complicates the comparison

even further. At mid-gestation the affected human foetus has high concentrations of copper in the placenta and all other organs except the liver, and especially high concentrations in the kidney (Heydorn *et al.*, 1975), whereas the affected mouse foetus at late gestation suffers from an overall copper deprivation (Table 4). Perhaps placental transfer of copper is more efficient in humans or else more efficient in mid-gestation compared with late gestation. However, the fact that serum copper can be normal at birth in affected human males (Grover & Henkin, 1976) suggests the former explanation. Biopsies in human patients performed in the first few months of life show low copper concentrations in the liver (Danks *et al.*, 1972) and elevated concentrations in the duodenal mucosa (Danks *et al.*, 1973). The general pattern of tissue concentrations observed at autopsy in humans (Grover & Scrutton, 1975; J. Camakaris, B. J. Stevens & A. Finch, unpublished work) parallels that seen in 11-day-old mutant mice. Consequently it does seem that the two organisms are showing a very similar general disturbance which may be somewhat altered by species differences.

The higher retention of copper by those tissues which express the mutation (e.g. gut, kidney) may be due to an alteration in a copper-binding protein which has resulted in its having a higher affinity for copper (Danks, 1975, 1977; Beratis *et al.*, 1978), or alternatively the mutation may have resulted in loss of function of a copper-binding protein or an enzyme which modifies such a protein so that copper then binds non-specifically to various intracellular proteins. The role of the liver and other tissues is discussed by Mann *et al.* (1979). Further work is required to compare the intracellular copper-binding components of mutant and normal cells and tissues. Identification of the primary defect in these mutants should provide much-needed fundamental knowledge on the normal cellular handling of copper.

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References

- Beratis, N. G., Price, P., LaBadie, G. & Hirschhorn, K. (1978) *Pediatr. Res.* **12**, 699-702
- Bingley, T. B. & Dick, A. T. (1969) *Clin. Chim. Acta* **25**, 480-482
- Brückmann, G. & Zondek, S. G. (1939) *Biochem. J.* **33**, 1845-1857
- Camakaris, J., Mann, J. R. & Danks, D. M. (1978) *Aust. Paediatr. J.* **14**, 115
- Clark, S. L., Jr. (1959) *J. Biophys. Biochem. Cytol.* **5**, 41-49
- Danks, D. M. (1975) *N. Engl. J. Med.* **293**, 1147-1149
- Danks, D. M. (1977) *Inorg. Perspect. Biol. Med.* **1**, 73-100

- Danks, D. M., Stevens, B. J., Campbell, P. E., Gillespie, J. M., Walker-Smith, J., Blomfield, J. & Turner, B. (1972) *Lancet* **i**, 1100-1103
- Danks, D. M., Cartwright, E., Stevens, B. J. & Townley, R. R. W. (1973) *Science* **179**, 1140-1142
- Dekaban, A. S., Aamodt, R., Rumble, W. F., Johnston, G. S. & O'Reilly, S. (1975) *Arch. Neurol. (Chicago)* **32**, 672-675
- Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167-182
- Edwards, K. D. G. & Whyte, H. M. (1959) *Australas. Ann. Med.* **8**, 218-224
- Evans, G. W. & Reis, B. L. (1978) *J. Nutr.* **108**, 554-560
- Evans, G. W. & Wiederanders, R. E. (1967) *Am. J. Physiol.* **213**, 1177-1182
- Fraser, A. S., Sobey, S. & Spicer, C. C. (1953) *J. Genet.* **51**, 217-221
- Grover, W. D. & Henkin, R. I. (1976) *Pediatr. Res.* **10**, 448
- Grover, W. D. & Scrutton, M. C. (1975) *J. Pediatr.* **86**, 216-220
- Heydorn, K., Damsgaard, E., Horn, N., Mikkelsen, M., Tygstrup, S., Vestermark, S. & Weber, J. (1975) *Humangenetik* **29**, 171-175
- Hunt, D. M. (1974) *Nature (London)* **249**, 852-854
- Hunt, D. M. (1976) *Life Sci.* **19**, 1913-1920
- Leblond, C. P. & Stevens, C. E. (1948) *Anat. Rec.* **100**, 357-377
- Mann, J. R., Camakaris, J., Danks, D. M. & Walliczek, E. G. (1979) *Biochem. J.* **180**, 605-612
- Mistilis, S. P. & Mearrick, I. T. (1969) *Scand. J. Gastroenterol.* **4**, 691-696
- Phillips, R. J. S. (1961) *Genet. Res.* **2**, 290-295
- Prins, H. W. & Van den Hamer, C. T. (1979) *Proc. Int. Symp. Trace Element Metabolism in Man and Animals* 3rd in the press
- Stevens, B. J. (1972) *Clin. Chem.* **18**, 1379-1384
- Vissek, W. J., Whitney, J. B., Kohn, J. S. G., III & Comar, C. L. (1953) *Proc. Soc. Exp. Biol. Med.* **84**, 610-615
- Volpintesta, E. J. (1974) *Am. J. Dis. Child.* **128**, 244-246
- Widdowson, E. M., Chan, H., Harrison, G. E. & Milner, R. D. G. (1972) *Biol. Neonate* **20**, 360-367