Copper Metabolism in Mottled Mouse Mutants

DISTRIBUTION OF ^{64}Cu IN BRINDLED (Mo^{br}) MICE

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1. Duodenal injection of ^{64}Cu in treated adult mutant mice ($\text{Mo}^{\text{br/y}}$) revealed severe malabsorption of copper. In suckling mutants, malabsorption was less severe, owing to delayed absorption between 2 and 5 h after injection. Pinocytosis at the distal small intestine seems the likely explanation for this difference, and this is supported by results of ileal injection of radioisotope in the suckling mice. 2. The distribution of ^{64}Cu in various organs was measured in suckling normal, mutant and heterozygote mice and in adult normal and mutant mice during 48 h after intracardiac injection. Excessive accumulation of radioisotope was observed in most extrahepatic organs of mutant and heterozygote mice and was most pronounced in kidney. This could not be explained by initial copper deficiency. The livers of suckling mutant and heterozygote mice lost radioisotope rapidly after normal initial uptake. This pattern was not seen in adult mutants.

The brindled gene is an allele at the sex-linked Mottled locus of the house mouse (Fraser & Sobey, 1953) and causes hemizygote-affected males ($\text{Mo}^{\text{br/y}}$) and homozygote-affected females ($\text{Mo}^{\text{br/br}}$) (Mann *et al.*, 1979) to die at around 14 days *post partum*. These mice are an excellent model for the human congenital copper-deficiency disease, Menkes' syndrome. The human and mouse mutations appear homologous.

Previous studies with copper isotopes in mottled mouse mutants have been limited: distribution of isotope was observed 48 h after administration via the drinking water in 28-day-old blotchy mice ($\text{Mo}^{\text{bl/y}}$) (Hunt, 1976) and 24 h after injection into the peritoneum of 3-day-old blotchy mice (Van den Hamer & Prins, 1978). Evans & Reis (1978) demonstrated defective absorption of radioisotope at 48 h by suckling brindled mice. Radioisotope was injected subcutaneously into the mother, allowing it to reach the suckling mice via maternal milk. This achieved optimal physiological conditions, but gave little control over the dose to each mouse. Calculations from the published data indicate that some of the observations reported must have been made close to the limit of reliable radioactivity counting rates.

The observation of low radioisotope content in mutant livers in all of these studies was described as impaired hepatic uptake. However, such observations made at a single time (24 or 48 h after administration) must represent the net effect of uptake, binding and release. Furthermore, interpretation of the distribution observed in internal organs was complicated by not

administering the radioisotope directly into blood. Copper would be malabsorbed by mutants even after subcutaneous and intraperitoneal injection, owing to the high retention of copper by mutant tissue (Mann *et al.*, 1979).

The present study describes the distribution of ^{64}Cu at serial time intervals to give an indication of the kinetic situation in regard to intestinal absorption and to distribution of body tissues. The latter was achieved by using intracardiac injection.

Experimental

Materials

Our Mo^{br} strain was kindly supplied by Dr. David Hunt, Queen Mary College, London. Mutant males ($\text{Mo}^{\text{br/y}}$) survived to adult life after an injection of $50\mu\text{g}$ of copper at 7 days of age (Mann *et al.*, 1979). These animals are healthy despite recurrence of copper deficiency.

Adult mice were fed *ad libitum* on Mecon rat and mouse cubes containing $16.7\mu\text{g}$ of copper/g dry wt. and acidified water containing $2.5\mu\text{g}$ of copper/ml. Suckling and adult mice used in this study were 10–12 and 60–120 days of age respectively.

$^{64}\text{CuCl}_2$ (half-life 12.8 h) was purchased from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. Radioactivity was measured by using an LKB Ultragamma counter programmed for automatic decay and background correction.

All injections were made with 10, 25 and 100 μ l SGE micro-syringes.

Methods

Absorption of ^{64}Cu from the intestine. Suckling and adult normal ($\text{Mo}^{+/y}$) and mutant ($\text{Mo}^{\text{br}/y}$) mice were used in these experiments. The following method was adopted after oral administration and gastric intubation proved unsatisfactory in the small suckling mice, the latter often causing gastric perforation.

Mice were starved, anaesthetized with diethyl ether, and the abdomen was opened by a ventral midline incision. Suckling mice were injected with 10 μ l of radioisotope solution into the lumen of the duodenum through the stomach and pyloric sphincter, or into the ileum directly so that absorption of radioisotope could be observed when it was placed immediately at the most active site of pinocytosis, which is the lower region of the small intestine (Clark, 1959; Camakaris *et al.*, 1979). Adult mice received 100 μ l of radioisotope solution into the duodenum only by the same method. The abdomen was closed with surgical sutures. Mice were placed in waxed paper cups which had a layer of filter paper covered by gauze to absorb urine and separate faeces. Suckling mice were then placed under an i.r. lamp for approx. 10 min to aid recovery from the procedure, which was sometimes lethal to mutant mice if warming was not used.

After the mice had been left for the desired length of time they were killed. The organs and tissues were removed, rinsed in non-radioactive 0.01 M- CuCl_2 in 0.9% NaCl and placed in tubes for radioactivity counting. Dissection instruments were rinsed in 1% EDTA and dissection paper was changed after each dissection to avoid cross-contamination. The total animal and its excretions were counted for radioactivity, results being expressed as a proportion of the total recovered dose for each animal. Tissues and organs assayed were whole blood, urine, unwashed gut (gut and luminal contents), faeces, liver, kidneys and remaining carcass. Blood was weighed, counted for radioactivity and the results were expressed as % of recovered dose/g, and urine was assayed by counting the radioactivity of the filter paper at the bottom of the paper cup as well as that of bladder plus contents.

Distribution of ^{64}Cu after intracardiac injection. Suckling normal ($\text{Mo}^{+/y}$, $+/+$) mutant ($\text{Mo}^{\text{br}/y}$) and heterozygote ($\text{Mo}^{\text{br}/+}$) mice and adult normal and mutant mice were used in these experiments. Adult mutant mice had been injected at 7 days of age with 50 μ g of Cu^+ (Mann *et al.*, 1979).

Intracardiac injection was adopted for suckling mice, as it proved impractical to inject intravenously in such small animals. This procedure was also used for adult mice. The mice were anaesthetized with diethyl ether and the skin above the rib-cage was

opened by a ventral midline incision. A needle was inserted through the rib-cage into the heart, and 5 and 10 μ l of radioisotope solution injected into suckling and adult mice respectively. To obtain an estimate of the amount of radioisotope excreted into the bile of suckling mice, the gut was ligated at the upper jejunal loop in some animals to separate the duodenum and stomach from the rest of the alimentary tract before intracardiac injection. This produced a reservoir for bile collection.

All subsequent procedures were equivalent to those in the absorption studies, except that organs and tissues counted for radioactivity were blood, urine, unwashed gut, faeces, liver, kidneys, spleen, lungs, heart, brain, total skin, thigh muscle and remaining carcass of suckling mice, and these tissues except skin, heart and carcass of adult mice. Results for adult mice were therefore related to injected dose rather than total recovered dose, as the whole animal and its excretions were not counted for radioactivity. For suckling mice subjected to gut ligation, the stomach and duodenum plus contents were assayed separately from the remainder of the gut. Attempts to determine labelled caeruloplasmin were made by scaling down the method of Van den Hamer & Prins (1978) to use 200 μ l of mouse plasma.

Results and Discussion

Intestinal absorption of $^{64}\text{CuCl}_2$

Results for suckling mice are shown in Table 1, parts (a) and (b). Table 1(c) presents results for adult mice.

Injection of the radioisotope into the duodenum demonstrated that absorption was abnormal by mutant mice of both age groups. However, age influenced the results obtained. Adult mutants absorbed approximately one fifth as much radioisotope as normals at 2 and 5 h after administration. In suckling mice there was a difference of similar magnitude in the proportion of the dose absorbed after 2 h, but by 5 h the total absorption had reached almost that attained by normal mice. Furthermore, the total proportion of the dose absorbed after 5 h was greater in both normal and mutant suckling mice than in normal adult mice.

Ileal injection was conducted only in suckling mice. The proportion of the dose absorbed is closely comparable with that found after duodenal injection whether one considers normal or mutant mice or results after 2 or 5 h.

The information documented about the distribution of the radioisotope expressed as % of absorbed dose (i.e. not including radioactivity in gut wall and luminal contents) among various organs is also important. The most useful comparison is between liver and extrahepatic tissues, with kidneys showing

Table 1. Absorption of ⁶⁴Cu injected into the intestine

Three normal and three mutant suckling mice were injected with ⁶⁴CuCl₂ (7.8–11.4 μ Ci of ⁶⁴Cu; 5.4 μ g of Cu) into (a) the ileum and killed at 2 and 5 h. Results are means $\pm$ s.d. of three observations. Two normal and two mutant adult mice were injected with ⁶⁴CuCl₂ (75.5–86.4 μ Ci of ⁶⁴Cu; 9.1 μ g of Cu) into (b) the duodenum and killed at 2 and 5 h. Results are the means of two observations. Faeces were counted for radioactivity, but never contained measurable radioactivity. Absorbed dose is total recovered radioactivity minus radioactivity in gut wall and luminal contents. Average recovery of the administered dose was 95.8 $\pm$ 5.15% for suckling mice, and 112 $\pm$ 1.92% for adult mice.

	(a)			(b)			(c)		
	2 h		5 h	2 h		5 h	2 h		5 h
Percentage of recovered dose	Mo ^{+/y}	Mo ^{br/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}	Mo ^{br/y}
Gut and luminal contents	84.36 $\pm$ 3.37	92.74 $\pm$ 4.13	83.66 $\pm$ 3.03	82.24 $\pm$ 3.13	77.58 $\pm$ 3.62	95.26 $\pm$ 2.24	76.46 $\pm$ 9.85	79.91 $\pm$ 14.67	0.15 $\pm$ 0.23
Urine	0.37 $\pm$ 0.18	0.21 $\pm$ 0.08	0.45 $\pm$ 0.24	0.78 $\pm$ 0.09	0.56 $\pm$ 0.21	0.27 $\pm$ 0.14	0.15 $\pm$ 0.23	1.31 $\pm$ 0.36	0.20 $\pm$ 0.07
Remaining carcass	15.27 $\pm$ 3.30	7.05 $\pm$ 4.23	15.89 $\pm$ 2.83	16.98 $\pm$ 3.05	21.86 $\pm$ 3.83	4.47 $\pm$ 2.36	23.39 $\pm$ 9.92	18.78 $\pm$ 14.95	1.05 $\pm$ 0.20
Total absorption	15.64	7.26	16.34	17.78	22.42	4.74	23.54	20.09	1.10 $\pm$ 0.05
Percentage of absorbed dose									
Liver	53.64 $\pm$ 1.18	25.82 $\pm$ 10.97	58.34 $\pm$ 1.03	45.99 $\pm$ 7.68	32.03 $\pm$ 9.97	6.59 $\pm$ 5.69	51.37 $\pm$ 5.71	11.85 $\pm$ 2.41	58.51 $\pm$ 46.41
Kidney	5.89 $\pm$ 1.77	15.98 $\pm$ 8.55	6.71 $\pm$ 1.14	12.91 $\pm$ 3.75	2.96 $\pm$ 0.52	9.07 $\pm$ 5.70	3.67 $\pm$ 0.60	7.60 $\pm$ 1.78	5.14 $\pm$ 22.73
Remaining carcass	38.10 $\pm$ 3.94	55.31 $\pm$ 7.15	32.20 $\pm$ 1.68	36.71 $\pm$ 3.62	62.51 $\pm$ 12.11	78.64 $\pm$ 11.51	44.32 $\pm$ 5.20	74.03 $\pm$ 5.98	33.92 $\pm$ 27.27

Fig. 1. Distribution of ⁶⁴Cu in suckling mice

Two normal (■—■), two mutant (●—●) and two heterozygote (△—△) mice for each time interval received an intracardiac injection of ⁶⁴CuCl₂ (2.7–4.6 μ Ci of ⁶⁴Cu; 0.5–0.9 μ g of Cu) and were killed at 10 min, 1 h, 2 h, 5 h or 10½ h after injection. Mice to be killed at 35 h and 48 h were placed back with the mother after injection. Results for mice killed at 35 h and 48 h are expressed as % of administered dose, because a total recovery of the dose was not possible, owing to urinary excretion. (a), Kidney; (b), skin; (c), brain; (d), liver (l), ligated duodenum and stomach (ds).

the most marked change. The relative proportions of radioisotope found in liver as compared with other organs was affected by the site of injection into the gut

and by the mutation. Injection into the ileum diverted more copper to extrahepatic tissues in both normal and mutant mice, whereas mutant mice had substantially more radioisotope in extrahepatic tissues than did normal mice, whether the two groups were compared after duodenal injection or after ileal injection.

It is apparent that the process of copper absorption by adult mice was severely affected by the mutation. However, interpretation of the results from suckling mice requires consideration of the process of pinocytosis as well as the specific copper-absorption process. Pinocytosis is present only up to 18 days of age in mice and is most active at the distal small intestine (Clark, 1959). Furthermore, Mistilis & Mearrick (1969) have shown that suckling rats absorb biliary ^{64}Cu by pinocytosis and that they build up a very large proportion of ^{64}Cu in the distal small intestine. The absorption of copper by pinocytosis at the distal small intestine of mutant mice was evident from our previous observations (Camakaris *et al.*, 1979; Mann *et al.*, 1979).

All the results of the present paper would seem to

be explained by postulating that the process of pinocytosis is either unaffected or relatively little affected by the mutation. The delay in absorption of radioisotope by suckling mutants would be explained if pinocytosis was always a slow process or if the mutation was slowing the rate of pinocytosis. A simple explanation of time elapsed for transit of the radioisotope from duodenum to ileum is not a satisfactory explanation for the delay, since the delay was observed even after ileal injection. The relatively rapid absorption from the ileum of the normal mice suggests either that the specific copper-absorptive process present in the upper small intestine was also active in the ileum, or that the difference in rate of absorption was reflecting the influence of the mutation on the process of pinocytosis.

Material that is taken up by pinocytosis is known to be carried via lymphatics and therefore does not enter the portal circulation to the liver (Clark, 1959). This would seem to explain the different organ distribution of radioisotope after ileal injection in both normal and mutant mice. The effect of the mutation in causing accumulation of copper in cells

Table 2. *Distribution of ^{64}Cu in suckling mice*

Two normal, two mutant and two heterozygote mice for each time interval received an intracardiac injection of $^{64}\text{CuCl}_2$ (2.7–4.6 μCi ; 0.5–0.9 μg of Cu) and were killed at 10 min, 1, 2, 5, 10½ and 21 h after injection. Mice killed at 35 and 48 h were placed back with the mother after injection. The means of two observations are expressed as % of recovered dose for mice at 10 min–21 h, and as % of administered dose for mice at 35 and 48 h, as urine radioactivity was not counted. Blood and muscle are % of dose/g. Average recovery of the administered dose, including organs in Fig. 1, was $99.25 \pm 7.95\%$. Faeces were counted for radioactivity, but never contained measurable radioactivity.

Time after injection	Tissue ...	Blood	Urine	Gut	Spleen	Lung	Heart	Muscle	Remaining carcass
10 min	Mo ^{+/y}	42.40	0.11	4.50	0.38	2.85	0.89	6.50	28.00
	Mo ^{br/y}	52.70	0.19	4.40	0.40	3.10	0.98	6.90	30.40
	Mo ^{br/+}	55.10	—	4.80	0.61	1.90	—	—	21.79
1 h	Mo ^{+/y}	7.25	0.15	7.35	0.49	2.90	1.66	5.10	27.95
	Mo ^{br/y}	8.45	0.32	7.70	0.63	2.40	1.04	4.70	29.70
	Mo ^{br/+}	—	—	—	—	—	—	—	—
2 h	Mo ^{+/y}	5.30	0.61	13.65	0.71	0.81	0.23	4.30	14.50
	Mo ^{br/y}	9.00	0.69	8.50	0.56	2.65	0.87	8.00	29.00
	Mo ^{br/+}	—	—	—	—	—	—	—	—
5 h	Mo ^{+/y}	7.35	0.55	16.40	0.45	1.40	0.79	3.40	24.05
	Mo ^{br/y}	9.85	0.42	9.40	0.71	2.70	0.82	7.80	34.45
	Mo ^{br/+}	6.61	0.77	22.98	0.67	1.65	0.78	—	28.87
10½ h	Mo ^{+/y}	4.60	0.43	13.40	0.61	0.61	0.40	3.40	13.90
	Mo ^{br/y}	8.05	0.77	13.95	0.84	2.04	0.98	6.80	31.50
	Mo ^{br/+}	1.05	0.83	25.68	0.47	1.91	—	—	31.96
21 h	Mo ^{+/y}	5.80	0.89	14.35	0.69	0.75	0.49	3.40	17.00
	Mo ^{br/y}	7.85	0.78	14.40	0.92	2.25	0.77	7.00	36.55
	Mo ^{br/+}	1.10	0.90	20.90	0.69	1.83	—	—	35.35
35 h	Mo ^{+/y}	6.92	—	10.51	0.41	0.83	0.69	3.22	17.62
	Mo ^{br/y}	7.12	—	21.03	0.41	2.10	1.10	6.97	37.87
	Mo ^{br/+}	7.57	—	17.92	0.34	1.68	0.93	—	39.41
48 h	Mo ^{+/y}	6.35	—	9.98	0.45	0.77	0.61	4.40	17.69
	Mo ^{br/y}	6.77	—	18.53	0.32	1.81	0.78	7.08	37.32
	Mo ^{br/+}	6.22	—	18.68	0.41	2.21	1.00	—	40.89

cultured from mice (Danks, 1977; Danks *et al.*, 1978) and on the retention of ⁶⁴Cu by thigh muscle (Mann *et al.*, 1979) would explain the greater increase and retention of the radioisotope distributed to the extrahepatic tissues of mutant mice. This point is developed in more detail below.

It is unlikely that the slower recovery from the injection procedure by suckling mutant mice contributed to the delayed absorption of radioisotope, as previous studies have shown that the content of ⁵¹Cr in the gut wall after 2 h was the same in both suckling normal and mutant mice after administration by the same procedure (Camakaris *et al.*, 1979).

It seems then that the specific process of copper absorption is severely affected by the mutation, that pinocytosis at the distal small intestine is relatively preserved and that the net effect is delayed and diminished absorption of copper in suckling mutant mice.

Distribution of ⁶⁴Cu after intracardiac injection

Figs. 1(a)–1(d) and Tables 2 and 3 show that the distribution of ⁶⁴Cu as a function of time was greatly altered in suckling mutant and heterozygote mice and in adult mutant mice. In all organs heterozygote mice showed the same trends as for mutant males. No striking differences from normal were seen in initial uptake in the various organs. However, very definite differences emerged with increasing time. The patterns allow classification of organs into three classes in suckling mice.

1. Kidney of mutants and heterozygotes continued to accumulate isotope excessively over the 48 h; gut, lung, heart, muscle, skin and carcass showed this trend less strikingly.

2. Brain and spleen showed no definite difference between groups.

3. Liver of mutants and heterozygotes lost radioisotope rapidly after apparently normal initial uptake.

The organs of adult mice showed these same trends, except normal liver lost as much radioisotope as mutant liver over the 80 h period.

The accumulation of radioisotope by organs of class 1, especially kidney, skin and carcass, indicates that these tissues had a large avidity for copper. This avidity is likely to represent the expression of the mutation and parallels the phenotype seen in cultured cells (Goka *et al.*, 1976; Horn, 1976; Danks, 1977; Beratis *et al.*, 1978; Danks *et al.*, 1978). This seems very likely for kidney and lung, as these organs are not initially copper-deficient. In fact kidney copper concentrations are initially higher in mutants and heterozygotes (Camakaris *et al.*, 1979; Mann *et al.*, 1979). Although it may be thought that the copper deficiency observed in the other tissues (Camakaris *et al.*, 1979; Mann *et al.*, 1979) may have contributed to radioisotope accumulation, there are two obser-

Table 3. Distribution of ⁶⁴Cu in adult mice

Two normal and two mutant mice for each time interval received an intracardiac injection of ⁶⁴CuCl₂ (40 μCi of ⁶⁴Cu; 10 μg of Cu) and were killed at ½, 3½, 10, 20 and 50 h. Mice killed at 80 h received 99.5 μCi of ⁶⁴Cu and 25 μg of Cu in 25 μl to allow for radioisotope decay. The means of two observations are expressed as % of administered dose: values for plasma and muscle are % of dose/g. Faeces were counted for radioactivity, but never contained measurable radioactivity.

Time after injection	½ h		3½ h		10 h		20 h		50 h		80 h	
	Mo ^{+/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}	Mo ^{+/y}	Mo ^{br/y}
Plasma	4.81	1.87	0.94	1.93	1.46	1.44	2.5	0.98	1.36	1.11	0.57	0.57
Gut	11.51	10.89	15.39	14.70	10.48	20.73	10.34	14.06	5.79	13.41	5.05	9.15
Liver	63.75	64.89	53.05	50.58	39.58	25.49	21.05	15.86	15.95	18.41	7.39	9.49
Kidney	1.85	7.88	1.50	11.09	2.60	14.89	2.49	22.19	2.37	13.48	1.63	13.12
Spleen	0.11	0.16	0.08	0.26	0.15	0.18	0.16	0.18	0.11	0.17	0.14	0.15
Lung	0.51	0.63	0.50	0.75	0.75	0.94	0.74	0.95	0.57	1.02	0.56	0.76
Brain	0.04	0.04	0.02	0.07	0.06	0.12	0.08	0.10	0.09	0.11	0.13	0.15
Muscle	0.35	0.35	0.16	0.34	0.24	0.43	0.29	0.51	0.42	0.66	0.49	0.58

Table 4. *Distribution of ^{64}Cu in suckling mice pretreated with copper*

Two normal and two mutant mice were injected subcutaneously with 50 μg of copper as CuCl_2 at 7 days, received an intracardiac injection of $^{64}\text{CuCl}_2$ (3.7 μCi of ^{64}Cu ; 0.5 μg of Cu) 3 days later and were killed after 21 h. A 50 μg injection of copper elevates copper contents of deficient organs of mutant mice to approximately normal values except for liver (Mann *et al.*, 1979). Results for copper-treated mice are means of two observations expressed as % of recovered dose. Results for untreated mice are from Figs. 1(a)–1(d) and Table 2. Values for blood are % of dose/g. Average recovery of the administered dose was $97.70 \pm 2.34\%$.

Tissue	$\text{Mo}^{+/y}$		$\text{Mo}^{\text{br}/y}$	
	Untreated	Cu-treated	Untreated	Cu-treated
Blood	5.80	4.67	7.85	4.77
Urine	0.89	0.48	0.78	0.44
Gut	14.35	7.33	14.40	14.98
Liver	54.00	73.08	18.65	43.70
Kidneys	1.45	0.82	8.00	4.76
Spleen	0.69	0.74	0.92	0.62
Lungs	0.75	0.48	2.25	1.65
Brain	1.05	0.44	0.91	0.37
Skin	8.20	5.51	15.30	9.17
Remaining carcass	17.00	10.24	36.55	23.77

variations which suggest that this was not a significant factor.

Firstly, heterozygote tissues accumulated radioisotope to the same extent as mutant tissue even though they possess higher copper concentrations than mutant hemizygotes (Camakaris *et al.*, 1979). Secondly, pre-injection of suckling mutants with 50 μg of copper elevated extrahepatic-tissue copper to approximately normal values (Mann *et al.*, 1979), but did not abolish the excessive accumulation of radioisotope in these organs (Table 4).

Any tendency toward accumulation of ^{64}Cu by brain of suckling mutants and heterozygote may have been prevented by the blood–brain barrier. This may have restricted entry of radioisotope, as the proportions taken up by normal, mutant and heterozygote brains were very low.

Figs. 1(a)–1(d) and Tables 2 and 3 show that initial rates of ^{64}Cu uptake appear to be normal in all organs of mutant and heterozygote mice. This includes the liver of suckling mutants, which demonstrates that this organ does not suffer from impaired copper uptake, as claimed by others (Hunt, 1976; Van den Hamer & Prins, 1978; Evans & Reis, 1978), but exhibits defective copper retention. This observation is believed to reflect true copper loss from the liver and not to be an artefact of smaller dilution owing to a low liver copper pool, because stored copper and newly taken up copper have been shown to behave as different pools (Hazelrig *et al.*, 1966; Owen & Orvis, 1970; Evans, 1973). A lack of copper retention has also been observed in the liver of suckling mutants after an injection of a large dose of copper subcutaneously (Mann *et al.*, 1979).

The radioisotope was not lost from the liver of suckling mutants and heterozygotes in bile, as measured in the ligated duodenum and stomach (Fig. 1d). Rather, the loss from the liver and the continued uptake in various extrahepatic tissues were related. Labelled caeruloplasmin was not detected by our method in normal or mutant suckling mice at 10 min, 5 and 21 h after injection.

Explanation of the total findings seems to require both excessive avidity of certain extrahepatic tissues for copper and a lack of retention of copper by the liver of suckling mutants. The lower ^{64}Cu retention by normal adult liver compared with the liver of normal suckling mice is believed to reflect the content of hepatic copper-binding sites at these ages (Evans, 1973). A lack of hepatic copper-binding sites may have been the cause of the defective ^{64}Cu retention by the liver of suckling mutants and heterozygotes.

It is possible that the primary defect is expressed by the liver and decreases its ability for copper binding. However, defective copper retention is the converse of the expression of the primary defect observed in cultured fibroblastic cells, renal-epithelial cells and probably many extrahepatic tissues *in vivo*, which is accumulation of excessive amounts of copper. Decreased exposure of the liver cells of suckling mutants to copper during prior development may have prevented the induction of copper-binding sites for long-term retention.

Final proof of the expression of the mutation and the nature of the basic defect in various tissues of the brindled mice would seem to lie with studies conducted with isolated tissues in culture and by fractionation of cellular copper-binding components.

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